CHARACTERIZATION OF THE REGULATORY MECHANISM CONTROLLING PHYTOTOXIN PRODUCTION BY *Pseudomonas syringae* pv. *syringae*

A Dissertation

by

NIAN WANG

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2005

Major Subject: Plant Pathology

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Approved by:

Chair of Committee,	Dennis C. Gross
Committee Members,	Carlos F. Gonzalez
	Jeffrey Z.Chen
	Michael V. Kolomiets
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ABSTRACT

Characterization of the Regulatory Mechanism Controlling Phytotoxin Production by *Pseudomonas syringae* pv. *syringae*. (December 2005)

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Syringopeptin (syp) and syringomycin (syr) are major necrosis-inducing lipodepsipeptide phytotoxins produced by *P. syringae* pv. *syringae*. This report demonstrates that syringopeptin production is activated by plant signal molecules. Syringopeptin production by BR132 was increased two-fold by addition of arbutin (100 μM) and D-fructose (0.1%) to syringomycin minimal medium (SRM). Subgenomic analysis of transcriptional expression with a 70-mer oligonucleotide microarray demonstrated that the *syr-syp* genes are induced 2.5- to 10.5-fold by arbutin and D-fructose. The *syr-syp* genomic island was found to be organized into 12 transcriptional units based on reverse transcriptional PCR (RT-PCR) and computer analysis. The transcriptional start sites of the *salA* gene and operons III and IV were located 63, 75, and 104-bp upstream of the start codons of *salA*, *syrP*, and *syrB1*, respectively, using primer extension analysis. The predicted -10/-35 promoter region of operon IV was confirmed based on mutagenesis analyses of the *syrB1*::*uidA* reporter with β-glucuronidase (GUS) assays. A 20-bp conserved sequence

(TGTCccgN4cggGACA) with dyad symmetry around the -35 region was identified via computer analysis for the *syr-syp* genes/operons responsible for biosynthesis and secretion of syringomycin and syringopeptin. Expression of the *syrB1*::*uidA* fusion was decreased 59% when 6-bp was deleted from the 5' end of the *syr-syp* box in the promoter region of operon IV. These results demonstrate that the conserved promoter sequences of the *syr-syp* genes contribute to the co-regulation of syringomycin and syringopeptin production. Microarray analysis established that the *syr-syp* genes responsible for synthesis and secretion of syringomycin and syringopeptin belong to the SyrF regulon. Vector pMEKm12 was successfully used to express both SalA and SyrF proteins fused to maltose-binding protein (MBP). Both MBP-SalA and MBP-SyrF fusion proteins were purified with maltose-affinity chromatography. Gel shift analysis revealed that the purified MBP-SyrF, but not the MBP-SalA fusion protein, bound to a 262-bp fragment containing the *syr-syp* box. Purified MBP-SalA caused the shift of a 324-bp band containing the putative *syrF* promoter. Gel filtration analysis or cross-linking experiments indicated that both SalA and SyrF form dimers in vitro. This study may provide an important perspective on the regulation of syringomycin and syringopeptin production.

DEDICATION

To my wife, Guoping Liu, my daughter Cindy Wang, and my parents, Shijin Wang and Anlan He.

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CHAPTER I

INTRODUCTION

Syringomycin and syringopeptin are important elements of virulence for most phytopathogenic strains of *Pseudomonas syringae* pv. *syringae*, a prevalent bacterial plant pathogen with the distinctive ability to cause necrotic infections on a broad range of monocot and dicot species (131). Both syringomycin and syringopeptin are synthesized by a nonribosomal mechanism of peptide biosynthesis (58, 64, 147, 177). The syringomycin (*syr*) and syringopeptin (*syp*) gene clusters are located adjacent to one another on the chromosome to constitute a 132-kb genomic island (64, 147, 148, 177). My goal is to resolve the regulatory mechanism that controls toxin production and expression of virulence genes during plant-microbe interactions. This study will contribute to a better understanding of the molecular basis of virulence of *P. syringae* pv. *syringae* and how virulence genes respond during the plant-pathogen interaction. Moreover, the regulatory mechanisms required for microbial phytotoxin production and virulence are largely unknown despite determinative effects on pathogenicity.

Genes identified to be directly involved in regulation, synthesis, and secretion of syringomycin and syringopeptin are present in a genomic island (148). The synthesis of syringomycin and syringopeptin utilizes large multifunctional peptide synthetases composed of one or more modules to catalyze the activation and addition of amino acids

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This dissertation follows the style of Journal of Bacteriology.

to the growing peptide chain (106). The synthetase genes for syringomycin include *syrB1* and *syrE*, which contain nine catalytic modules. The synthetase genes of syringopeptin consist of *sypA*, *sypB*, and *sypC*, which contain 22 catalytic modules (64, 116, 147, 177). The *syr* and *syp* gene clusters also include three regulatory genes: *salA*, *syrF*, and *syrG* and nine genes (i.e., *syrD*, *syrP*, *pseA*, *pseB*, *pseC*, two *mtrC* homologs, one *oprM*, and one ORF encoding a putative threonine efflux protein) dedicated to secretion of the two toxins (83, 101, 132, 147, 148).

To grow in the apoplast, *P. syringae* senses its environment and induces genes required for host infection. As a result, the pathogen adapts to the *in planta* environment and modulates the physiology of the host (5, 5). Previous studies showed that many known *P. syringae* pathogenicity and virulence determinants, including the *hrp*/*hrc* genes, *avr* genes, and virulence genes that are dedicated to the production of syringomycin and coronatine, are induced upon inoculation into plant tissues (5, 18, 114, 115, 131, 170).

Production of syringomycin is modulated by perception of plant signal molecules (114, 115, 131). The primary signals are specific phenolic glycosides that are abundant in the tissues of many plant species. This was demonstrated by the induction of β-galactosidase activity resulting from a transcriptional fusion between *syrB1* and a promoterless *lac* operon as a reporter of gene activity in strain B3AR132 (115). In a survey of 34 plant phenolic compounds, only certain phenolic glucosides exhibited *syrB1*-inducing activity, including arbutin, phenyl-β-D-glucopyranoside, salicin, and esculin. Arbutin was shown to be the most efficient molecule for induction of *syrB1*.

The plant signal molecules that activated toxin production by *P. syringae* pv. *syringae* are distinct from those that induce the *vir* genes of *Agrobacterium tumefaciens* and the *nod* genes of rhizobia (126). All *syrB*-inducing phenolic signal molecules are characteristic of an intact glucosidic linkage. Sugars that occur in large quantities in leaf tissue markedly enhanced phenolic signal activity (114, 115). D-fructose is the most active sugar, stimulating a 10-fold increase of signal activity when phenolic signals occur at low concentrations (i.e., 1 to 10 μM) in strain B3AR132. D-fructose and sucrose also exhibit intrinsic low-level *syrB1*-inducing signal activity in the absence of the phenolic inducer. I predicted that other genes associated with syringomycin synthesis, secretion, or regulation are induced by plant signal molecules. Since syringopeptin is another important lipopeptide toxin produced by *P. syringae* pv. *syringae* and the *syp* genes belong to one genomic island together with *syr* genes, it is hypothesized that *syp* genes are also induced by plant signals.

The mechanisms by which *P. syringae* pv. *syringae* recognizes the plant signal molecules remain to be determined. It was reported that amphisin, a cyclic lipopeptide produced by *Pseudomonas sp*. strain DSS73, was regulated by components of sugar beet seed exudates via the GacS/A two component system (89). It is expected that syringomycin and syringopeptin production in *P. syringae* pv. *syringae* are modulated by plant signals via the GacS/A pathway as well.

In addition to the induction of virulence factors in plant-microbe interactions, most *hrp*/*hrc*, and avirulence (*avr*) genes of *P. syringae* are also activated during both compatible and incompatible interactions (133). The *hrp*/*hrc* genes are conserved in

phytopathogenic bacteria and affect the ability of the bacteria to induce a hypersensitive response (HR) in nonhost plants, as well as growth and pathogenicity in host plants (154). A Type III protein secretion apparatus encoded by the *hrp*/*hrc* genes mediates the transfer of bacterial proteins into the cytosol of host cells, where they modulate normal host cell processes (154). The majority of known *P. syringae* effector genes are able to elicit host defense responses that prevent disease in some plant hosts (170). Consequently, these genes are referred to as *avr* genes. These proteins may promote parasitism and disease and contribute to virulence of *P. syringae* in a susceptible plant-microbe interaction (28, 65, 170). In *P. syringae* pv. *syringae* 61, expression of *hrp* and *avr* genes is regulated by a multicomponent system including *hrpR, hrpS,* and *hrpL* (99). HrpS and HrpR proteins coordinate in conjunction with RpoN to activate the promoter of *hrpL*, the alternative sigma factor for *hrp* and *avr* genes (78). It remains to be determined what plant signals induce *hrp* and *avr* genes and how *P. syringae* pv. *syringae* recognizes the plant signal molecules.

The *salA* and *syrF* regulatory genes are required for regulation of syringomycin production and virulence of *P. syringae* pv. *syringae* (101). It appears that the GacS/A system is at the start of the regulatory hierarchy controlling syringomycin. The *gacS*/*A* two-component signal transduction system positively regulates expression of the *salA* gene, and *salA* gene is required for expression of the *syrB1* synthetase gene of syringomycin (88). I predict that the production of syringomycin and syringopeptin is co-regulated by the same mechanism and the syntheses of both toxins are positively controlled by *gacS*/*A*, *salA*, and *syrF*.

It is hypothesized that the interactions between the promoters and the regulators, including SyrF and certain sigma factors, determine the co-regulation of syringomycin and syringopeptin. The SyrF and SalA proteins share high similarity to LuxR regulatory family proteins, such as GerE (40) and FixJ (9). Both SalA and SyrF proteins contain helix-turn-helix (HTH) DNA–binding domains at the C-termini (101). I predicated that SalA and SyrF resemble the LuxR proteins in functions. Since SalA controls the expression of *syrF* (101), it is expected that SyrF will bind to the promoter regions of *syr-syp* genes directly. It remains to be understood how SalA controls *syrF*. I expected that the *syr-syp* genes should share conserved promoters for co-regulation.

The production of syringomycin and syringopeptin is crucial to the plant-pathogen interaction. The delicate coordination between the virulence and pathogenicity factors plays a key role in the invasion of the plant by the pathogens. The regulatory mechanism of virulence factors is largely unknown despite their importance. How dose *P. syringae* pv. *syringae* sense the environmental signals and activate the virulence and pathogenicity-related genes for invasion of the hosts? Is syringopeptin production regulated by the same mechanism as syringomycin? If so, what is the mechanism that co-regulates expression of syringomycin and syringopeptin synthesis? Do the *syr-syp* genes share conserved promoters? What are the roles of SalA and SyrF in the regulation of syringomycin and syringopeptin biosynthesis? I believe that it is important to answer these questions in order to determine the regulatory mechanism controlling virulence and pathogenicity-related genes with significant roles in the plant-pathogen interactions. The specific objectives of my proposed research are (i) to

characterize how plant signals affect the expression of the *syr-syp* genes and other virulence and pathogenicity-related genes, (ii) to characterize the promoters of the *syr-syp* genes and the sigma factor required for their transcription, and (iii) to determine the roles of SalA and SyrF in the regulation of syringomycin and syringopeptin production.

CHAPTER II

THE COORDINATED EXPRESSION OF GENES ENCODING LIPODEPSIPEPTIDE PHYTOTOXINS IN RESPONSE TO PLANT SIGNAL MOLECULES BY *P. syringae* pv. *syringae*

OVERVIEW

Specific plant signal molecules are known to induce syringomycin production and expression of *syrB1*, a syringomycin synthetase gene, in *P. syringae* pv. *syringae*. This report demonstrates that syringopeptin production likewise is activated by plant signal molecules and that the GacS, SalA, and SyrF regulatory pathway mediates transmission of plant signal molecules to the *syr-syp* biosynthesis apparatus. Syringopeptin production by BR132 was increased two-fold by addition of arbutin (100 μM) and D-fructose (0.1%) to syringomycin minimal medium (SRM). Among 10 plant phenolic compounds tested, only the phenolic glucosides arbutin, salicin, and phenyl-β-D-glucopyranoside substantially induced the β-glucuronidase (GUS) activity of a *sypA*::*uidA* reporter from 242 U per 10^8 CFU without plant signal molecules up to 419 U per 10^8 CFU with plant signal molecules. Syringopeptin production was found to be controlled by the SalA/SyrF regulon because no phytotoxin was produced by B301DSL7 (i.e., *salA* mutant) and B301DSL1 (i.e., *syrF* mutant), and the expression of *sypA*::*uidA* was decreased by 99% and 94% in *salA* (B301DSL30) and *syrF* (B301DNW31) mutant backgrounds, respectively. Subgenomic analysis of transcriptional expression with a 70-mer oligonucleotide microarray demonstrated that

the *syr-syp* genes were induced 2.5- to 10.5-fold by arbutin and D-fructose. This study establishes that plant signal molecules are transmitted through the GacS, SalA/SyrF pathway to activate the coordinated transcriptional expression of the *syr-syp* genes.

INTRODUCTION

Bacteria commonly detect specific plant metabolites, which serve as signals for regulating expression of virulence genes that allow adaptation to the plant environment. For example, several virulence (*vir*) genes in *Agrobacterium tumefaciens* are transcriptionally activated by specific phenolic signal molecules such as acetosyringone (111). The induction is enhanced by pyranose sugars such as D-fructose, D-mannose, and D-glucose at low concentrations. Accordingly, the inducing sugars enhanced the expression of a *virB*::*lacZ* fusion more than 10-fold at a 10 mM concentration in the presence of 2.5 μM acetosyringone (8, 150). Similarly, plant signal molecules have been reported to activate the expression of genes involved in toxin synthesis (i.e., syringomycin, coronatine), cell wall degradation (i.e., *pel*), and the Hrp Type III secretion system (12, 97, 115, 131, 173). Thus, plant signal molecules appear to play an important role in the interaction between plants and bacteria.

Syringomycin production is modulated by the perception of specific plant signal molecules (114, 115, 131). Syringomycin is one of the major virulence factors of *P. syringae* pv. *syringae*, which incites stem and leaf diseases in numerous monocot and dicot plants throughout temperate regions of the world (146). Inoculations of immature cherry fruits demonstrate a rapid and strong expression of *syrB1*, a synthetase gene for syringomycin (115). Cherry tissues contain plant signal molecules, such as flavonol

glycosides (quercetin and kaempferol 3-rutinosyl-4'-glucoside) and a flavanone glucoside (dihydrowogonin 7-glucoside), that are able to transcriptionally activate expression of a *syrB1*::*lacZ* fusion (114). In a survey of 34 phenolic compounds, only specific phenolic glycosides exhibited *syrB1*-inducing activity. The phenolic glycosides that exhibited *syrB1*-inducing activity included arbutin, salicin, and esculin, which are abundant in the tissues of many plant species (115). Arbutin was shown to be an efficient signal molecule that induced the high expression of the *syrB1* synthetase gene (115). The plant signal molecules that activate toxin production by *P. syringae* pv. *syringae* are different from those that induce the *vir* genes of *A. tumefaciens* and the *nod* genes of rhizobia (111, 112, 126). All *syrB1*-inducing phenolic signal molecules characteristically contain an intact glycosidic linkage. Specific sugars that occur in large quantities in leaf tissues markedly enhance phenolic signal activity (114, 115). D-fructose is the most active sugar, causing a 10-fold stimulation of signal activity in strain B3AR132 when phenolic signals are present at low concentrations (i.e., 1 to 10 μM). D-fructose and sucrose also exhibit intrinsic low-level *syrB1*-inducing signal activity in the absence of the phenolic inducer. Certain strains of the bacterium, such as B3A, require plant signal molecules for syringomycin production grown under defined cultural conditions. Nearly all *P. syringae* pv. *syringae* strains produce higher syringomycin levels when the plant signal molecules arbutin and D-fructose are added to the media, but the extent of induction varies for individual strains. Several strains produce more than 10-fold higher toxin levels in the presence of arbutin and D-fructose, while other strains, including B301D, show approximately a 2-fold increase of

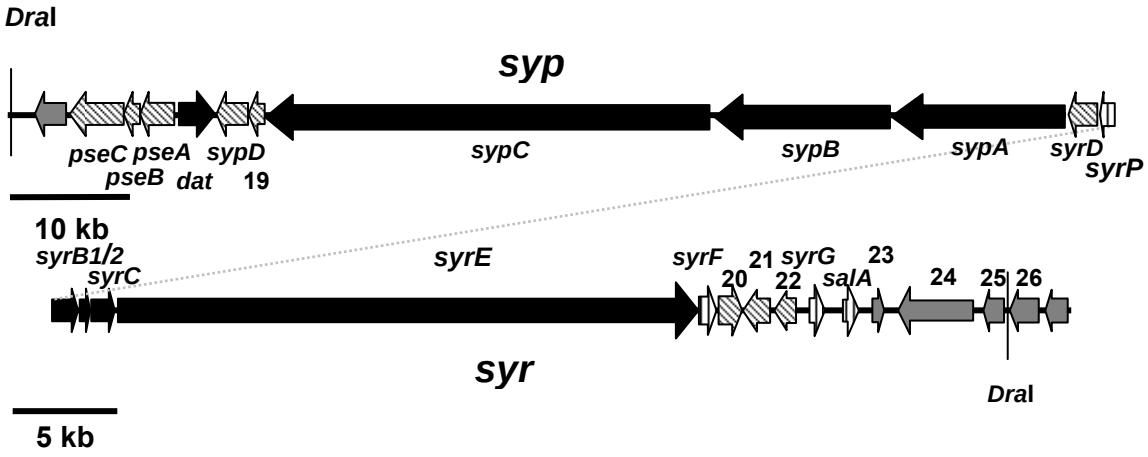


FIG. 2.1. A physical map of a 132-kb genomic DNA region of *P. syringae* pv. *syringae* strain B301D containing both syringomycin (*syr*) and syringopeptin (*syp*) gene clusters. The positions and orientations of the known and potential open reading frames (ORFs) are shown as horizontal arrows. The solid, diagonally-striped, and vertically-striped arrows represent genes that are predicted to be involved, respectively, in the synthesis, regulation, and secretion of the phytotoxins. The gray arrows represent the potential ORFs for which functions remain unknown.

syringomycin production (131).

Syringopeptin is another lipodepsipeptide that contributes to virulence of *P. syringae* pv. *syringae* (146). Syringopeptin and syringomycin have similar structures consisting of cyclic peptide heads attached to 3-hydroxy fatty acid tails. Both syringomycin and syringopeptin are synthesized by a nonribosomal mechanism of peptide biosynthesis (59, 148). The syringomycin (*syr*) and syringopeptin (*syp*) gene clusters (Fig. 2.1) are located adjacent to one another on the chromosome and are approximately 42-kb and 90-kb in size, respectively (147). The *syr* and *syp* gene clusters include biosynthesis, regulatory, and secretion genes for syringomycin and syringopeptin production (83, 101, 132, 147, 148). Both syringomycin and syringopeptin production are controlled by the *salA* gene. The *syr-syp* genes belong to the SalA regulon based on microarray analysis (103). Microarray analysis is applicable to studies of the transcriptional regulation of the *syr-syp* genes under the same defined environmental conditions (103). *P. syringae* utilizes a well-tuned regulatory and signal transduction system to coordinate the expression of virulence factors in order to adapt to the plant environment. The *gacS/A* two component signal transduction system is well suited to sense and recognize environmental signals and to transduce these signals to downstream regulators (70). GacS is a transmembrane protein which functions as a histidine protein kinase that undergoes phosphorylation in response to environmental stimuli (76). GacA is a response regulator protein that is phosphorylated by GacS (70, 136). The GacS/A system is highly conserved in bacteria (70) and is dedicated to a variety of functions, such as pathogenicity (76), quorum sensing (25), secondary

metabolites (34), and biofilm formation (123). It was reported that production of the lipopeptide amphisin by *Pseudomonas* sp. strain DSS73 is regulated by components of sugar beet seed exudates via the GacS/A two component system (89). Mutational inactivation of either GacS or GacA abolishes the response of exoproduct genes such as *P. fluorescens* CHA0 *hcnA* (for HCN synthetase) to a solvent-extractable signal (69). The GacS/A two component signal transduction system positively regulates expression of the *salA* gene, which is in turn required for expression of *syrF* (101). The regulatory gene *syrF* positively controls the expression of the syringomycin *syrB1* synthetase gene (101). Previous studies showed that *salA* also is required for syringopeptin production, based on toxin bioassays, microarray analysis, and β-glucuronidase (GUS) assays (88, 103). Chatterjee et al. (2003) showed that GacA is a master regulator controlling *P. syringae* pv. *tomato* DC3000 *hrpR*, *hrpS*, and *hrpL*, which are required for the activation of Hrp Type III secretion and effector genes. GacA is apparently not required for *hrp* expression in *P. syringae* pv. *syringae* B728a (27). Based on the high similarity between *P. syringae* pv. *syringae* B728a and B301D, it was predicted that GacS/A is involved in the signal transduction of the B301D *syr-syp* genes, but not the *hrp* genes.

Despite progress made toward understanding the regulation of the *syr* and *hrp* genes, little is known about the regulation of syringopeptin or the signal transduction pathway of the *syr-syp* and *hrp* genes in *P. syringae* pv. *syringae* B301D. In this study, it was hypothesized that specific plant signal molecules induce the coordinated expression of the *syr-syp* and *hrp* genes of *P. syringae* pv. *syringae* and that these two categories of genes use different signal transduction pathways. To test this hypothesis, a

subgenomic 70-mer oligonucleotide microarray was used to measure the transcriptional profile of B301D grown on SRM agar medium as compared to SRM agar medium supplemented with arbutin and D-fructose (i.e., SRM_{AF}). Evidence is presented that the addition of arbutin and D-fructose resulted in significant increases in transcriptional levels of the genes contributing to synthesis, secretion and regulation of both syringomycin and syringopeptin, as well as the *hrp* genes. Mutational analysis of *gacS* shows that the GacS/A two component system is involved in the signal transduction of the *syr-syp* genomic island but not representative genes associated with the Hrp Type III secretion system. These results are discussed in regard to the involvement of the *gacS/A* two component system and two key regulatory genes, *salA* and *syrF*, in mediating plant signal transduction responsible for the transcriptional activity of genes associated with lipodepsipeptide phytotoxin production.

RESULTS

Induction of syringopeptin production by plant signal molecules

Syringopeptin production was increased substantially for derivatives of strains B301D-R and B3A-R (i.e., *syrB1* mutants BR132 and B3AR132, Table 2.1), respectively, in the presence of arbutin (100 μM) and fructose (0.1%) as plant signal molecules. B3A132 did not produce a visible zone of syringopeptin inhibitory to *Bacillus megaterium* Km growth on SRM agar medium, but the radius of the inhibitory zones were about 4.7 mm on SRM_{AF} agar medium (Fig. 2.2). Addition of arbutin and fructose increased the radius of the inhibitory zone produced by BR132 by 4 mm, representing a 66.7% increase in size. Similarly, no detectable zone of syringomycin

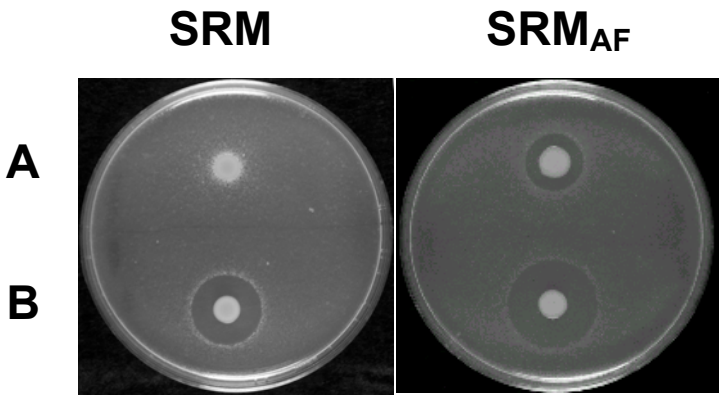


FIG.2.2. Bioassay for syringopeptin production by two strains of *P. syringae* pv. *syringae* grown on syringomycin minimal media without (SRM) or with 100 μM arbutin and 0.1% D-fructose (SRM_{AF}) as shown by zones inhibitory to *Bacillus megaterium*. The strains were grown 4 days on plates at 25°C. Plates were oversprayed with *B. megaterium* and allowed to incubate for 24 h at 25°C. Zones of inhibition indicate production of syringopeptin. A, B3AR132 (*syrB1* mutant); B, BR132 (*syrB1* mutant).

TABLE 2.1. Strains and plasmids

Strain or plasmid	Relevant characteristics	Source
Escherichia coli		
DH10B	F- *mcrA* Δ*lacX74* (ϕ80d*lacZ*ΔM15) Δ(mrr-*hsdRMS-mcrB*) *deoR recA1 endA1 araD139* Δ(*ara, leu*)7697 *galU galK* λ⁻ *rpsL nupG*	(57)
P. syringae pv. *syringae*		
B301D	Wild type, from pear	(30)
B301D-R	Spontaneous Rif^r derivative of B301D	(115)
B301DG12	*gacS*::*nptII* derivative of B301D; Km^r	(83)
B301DSL1	*syrF*::*nptII* derivative of B301D; Km^r	(101)
B301DSL7	*salA*::*nptII* derivative of B301D; Km^r	(101)
B301DSL6	*syrG*::*nptII* derivative of B301D; Km^r	(101)
B301DSL8	*syrB1*::*uidA-aaaC1*, derivative of B301D; Gm^r	(101)
B301DSL29	*sypA*::*uidA-aaaC1*, derivative of B301D; Gm^r	(103)
B301DSL30	*salA*::*nptII* derivative of B301DSL29; Gm^r Km^r	(101)
B301DNW31	*syrF*::*nptII* derivative of B301DSL29; Gm^r Km^r	This study
BR132	*syrB1::*Tn*3*HoHo1 derivative of B301D-R; Pip^r Rif^r	(115)
B3A	Wild type	(115)
B3A-R	Spontaneous Rif^r derivative of B3A	(115)
B3AR132	*syrB1*::Tn*3*HoHo1 derivative of B3A-R; Pip^r Rif^r	(115)
Plasmid		
pBluescriptSK(+)	Cloning vector; Ap^r	Strategene. La Jolla, CA
pUCP26	Cloning vector; Tc^r Ap^r	(169)
pBR325	Cloning vector; Cm^r Tc^r Ap^r	(128)
pLAFR3	Cloning vector; Tc^r	(153)
pNWE32	pGEM-T easy carrying *syrF*::*nptII* cloned from B301DNW31	This study
p29	pLAFR3 carrying a 16-kb fragment of B301D genomic DNA; Tc^r	(101)
pSL2	pBI101 with the 0.85-kb *aacC1* gene of pUCGM inserted at the *Eco*RI site downstream of the *uidA* gene; Km^r Gm^r	(101)
pSL19	pBR325 carrying the 5.8-kb *Bam*HI-*Kpn*I fragment of p29 with *nptII* insertion at *Bst*EII site of *syrF*; Ap^r Tc^r Km^r	(101)
pSL9	pBluescriptSK(+) carrying a 2.5-kb *kb Hin*dIII-*Eco*RV fragment of pSL5 containing *syrG*; Ap^r	(101)
pSL40F	pLAFR3 carrying the 3.0-kb *Eco*RI fragment of p29 with the 3.2-kb *uidA-aacC1* fragment from pSL2 inserted at the *Kpn*I site in-frame of *salA* in forward orientation; Tc^r Gm^r	(101)
pSL39F	pLAFR3 carrying the 2.5-*kb Hin*dIII-*Eco*RV fragment of pSL9 with the 3.2-kb *uidA-aacC1* fragment from pSL2 inserted at the *Eco*RI site in-frame of *syrG* in forward orientation; Tc^r Gm^r	(101)
pSL38F	pLAFR3 carrying the 5.8-kb *Bam*HI-*Kpn*I fragment of p29 with the 3.2-kb *uidA-aacC1* fragment from pSL2 inserted at the *Bst*EII site in-frame of *syrF* in forward orientation; Tc^r Gm^r	(101)

TABLE 2.2. Effects of plant phenolic β-glycosides on induction of *syrB1*::*uidA* and *sypA*::*uidA* fusions in strains B301DSL29 and B301DSL8, respectively

Phenolics added to SRM medium[a]	*syrB1*::*uidA*		*sypA*::*uidA*	
	GUS activity (U/10^8 CFU ± SE)[b,c]	Fold increase	GUS activity (U/10^8CFU ± SE)[b,c]	Fold increase
None	685 ± 63^A	--	242 ±14^A	--
Arbutin	1140 ± 73^B	1.66	419 ± 30^B	1.73
Phenyl-β-D-glucopyranoside	1050 ± 44^B	1.53	389 ± 24^B	1.60
Salicin	1060 ± 32^B	1.55	369 ± 18^B	1.52
Hydroquinone	755 ± 48^A	1.1	298 ± 19^A	1.23
Saligenin	732 ± 33^A	1.07	279 ± 27^A	1.15
Naringenin	710 ± 36^A	1.04	273 ± 23^A	1.13
Phenol	698 ± 51^A	1.02	269 ± 22^A	1.11
Quercetin	667 ± 23^A	0.97	268 ± 29^A	1.10
Phenyl-β-D-galactopyranoside	638 ± 57^A	0.93	252 ± 33^A	1.04
Rutin	686 ± 40^A	1.00	241 ± 22^A	0.99

[a] All phenolic compounds tested at a final concentration of 100 μM in SRM medium.

[b] Activities are the mean of six independent assays followed by the standard error of mean.

[c] Different letters indicate statistically significant differences (α=0.01). Differences between treatments were determined by Tukey's analysis of variance.

inhibitory to *Geotrichum candidum* was observed for B3A in the absence of plant signal molecules, but a small zone of syringomycin was observed on SRM_{AF} agar medium. The sizes of the zones of syringomycin production by B301D were 55.6% larger on SRM_{AF} agar medium than on SRM agar medium. Consistent results were obtained with repeats on three independent occasions.

Induction of *sypA*

The expression of *syrB1*::*uidA* fusion strain B301DSL8 was increased 1.66, 1.53, and 1.55-fold by addition 100 μM phenolic β-glucosides arbutin, phenyl-β-D-glucopyranoside, and salicin to SRM liquid medium, respectively (Table 2.2). Similarly, the expression of *sypA*::*uidA* fusion strain (B301DSL29) was increased 1.52- to 1.73-fold on SRM medium with the phenolic glucosides (Table 2.2). Arbutin was the most active inducer for both genes, increasing expression 1.73- and 1.66-fold, respectively (Table 2.2). Other phenolic compounds (hydroquinone, naringenin, phenol, phenyl-β-D-galactopyranoside, quercetin, rutin, and saligenin) tested did not show significant effect on expression of either *syrB1*::*uidA* or *sypA*::*uidA* fusions.

A β-glucosidic linkage was shown to be critical to signal activity. This was evaluated by testing the substituted derivatives of arbutin, phenyl-β-D-glucopyranoside and salicin (hydroquinone, saligenin, and phenol) (Table 2.2). All three phenolic aglucones (hydroquinone, saligenin, and phenol) were inactive in induction of both *syrB1*::*uidA* and *sypA*::*uidA* fusions.

Once it was confirmed that certain phenolic β-glycosides induced the expression of *sypA*, the effects of the sugar D-fructose on arbutin-mediated induction of *sypA* and

syrB1 were determined. Strain B301DSL8, which carries the *syrB1*::*uidA* fusion, produced high levels of GUS activity, averaging 1274 units per 10^8 CFU 3 days after incubation at 25°C in SRM_{AF} liquid medium. Without plant signal molecules, GUS activity of B301DSL8 averaged only 685 units per 10^8 CFU. Similarly, the GUS activity of B301DSL29, which contains the *sypA*::*uidA* reporter, was increased from 242 to 528 units per 10^8 CFU in the presence of arbutin (100 μM) and fructose (0.1%). Consequently, the expression of *syrB1* and *sypA* were increased 1.9- and 2.1-fold, respectively, by addition of arbutin and fructose to SRM liquid medium.

The *salA* and *syrF* regulatory pathway mediates induction of syringopeptin production and *sypA* expression

Neither B301DSL7, a *salA* mutant, nor B301DSL1, a *syrF* mutant, produced detectable syringopeptin zones of inhibition to *B. megaterium* Km on SRM or SRM_{AF} agar media (Fig. 2.3). B301DSL6, a *syrG* mutant, did not produce detectable zones of inhibition SRM agar medium, but did produce syringopeptin on SRM_{AF} agar medium (Fig. 2.3). Strain B301DSL29, carrying the *sypA*::*uidA* reporter, produced 242 and 528 GUS units per 10^8 CFU in SRM and SRM_{AF} liquid media, respectively, 3 days after incubation at 25°C. Expression of the *sypA*::*uidA* reporter was reduced approximately 99% in *salA* strain B301DSL30 (103), in both SRM and SRM_{AF} liquid media. Expression of the *sypA*::*uidA* reporter was decreased by 93.7% and 96.4% in *syrF* strain B301DNW31 in both SRM and SRM_{AF} media, respectively. Neither the *salA* mutant B301DSL30 nor the *syrF* mutant B301DNW31 exhibited a significant increase in GUS activity upon the addition of plant signal molecules (Table 2.3).

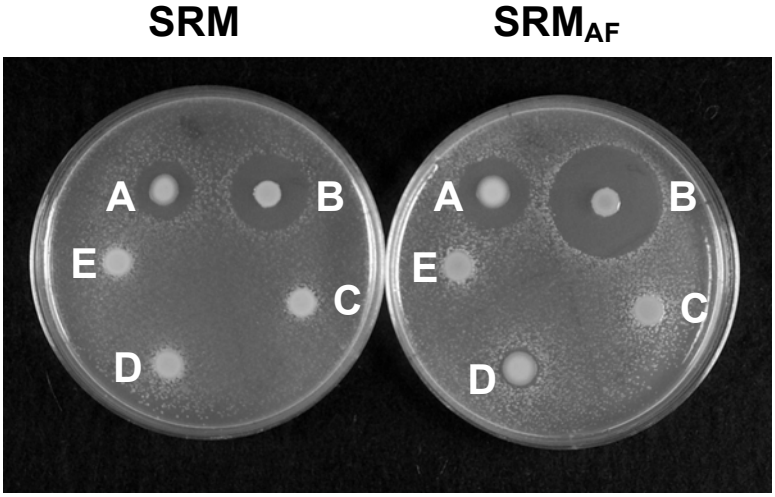


FIG. 2.3. Bioassay for syringopeptin production by different strains of *P. syringae* pv. *syringae* B301D grown on syringomycin minimal media without (SRM) or with 100 μM arbutin and 0.1% D-fructose (SRM_{AF}) as shown by zones inhibitory to *Bacillus megaterium.* The strains were grown on plates at 25°C for 4 days. Plates were oversprayed with *B. megaterium* and allowed to incubate for 24 h at 25°C. Zones of inhibition indicate production of syringopeptin. A, B301D; B, BR132 (*syrB1* mutant); C, B301DSL7 (*salA* mutant); D, B301DSL6 (*syrG* mutant); E, B301DSL1 (*syrF* mutant).

TABLE 2.3. Effects of *salA* and *syrF* mutations on expression of *sypA*::*uidA* expression in SRM liquid medium and SRM medium containing arbutin and D-fructose

Strain	Genotype	β-Glucuronidase activity ($U/10^8$ CFU ± SE)[a]	
		SRM	SRM_{AF}[b]
B301DSL29	*sypA*::*uidA*	242 ± 14	528 ± 37
B301DSL30	*sypA*::*uidA*; *salA*::*nptII*	1.9 ± 0.2	2.5 ± 0.7
B301DNW31	*sypA*::*uidA*; *syrF*::*nptII*	15.3 ± 0.5	18.8 ± 1.3

[a] Activities are means of six independent assays followed by the standard error of mean.

[b] Arbutin and D-fructose were added to SRM medium at a concentration of 100 μM and 0.1%, respectively.

TABLE 2.4. Microarray analysis of the stimulon controlled by plant signal molecules

Genes	Ratio (S.E.M)	Gene product	References
syrD	4.71 (±0.40)	ATP-binding secretion protein	(132)
syrP	7.76 (±0.61)	Homologue of histidine kinase	(178)
syrB1	5.23 (±0.40)	Syringomycin biosynthesis enzyme	(177)
syrB2	5.41 (±0.80)	Syringomycin biosynthesis enzyme	(177)
syrC	10.51 (±0.66)	Syringomycin biosynthesis enzyme	(177)
syrE-1	4.40 (±0.55)	Syringomycin synthetase	(64)
syrE-2	6.99 (±0.83)	Syringomycin synthetase	(64)
syrE-3	8.03 (±0.89)	Syringomycin synthetase	(64)
syrF	3.89 (±0.81)	LuxR family bacterial regulator	(101)
ORF20	6.18 (±0.93)	Putative out membrane protein	(103)
ORF21	2.53 (±0.48)	Hypothetical protein	(103)
ORF22	6.45 (±1.29)	Membrane protein	(103)
syrG	3.08 (±0.69)	LuxR family regulatory protein	(101)
salA	2.50 (±0.19)	LuxR family bacterial regulator	(101)
ORF23	0.99 (±0.07)	Hypothetical protein	(103)
ORF24-1	0.85 (±0.13)	Hypothetical protein	(103)
ORF24-2	0.92 (±0.03)	Hypothetical protein	(103)
ORF25	0.94 (±0.20)	Hypothetical protein	(103)
ORF26	1.02 (±0.14)	unknown (chemotaxis protein)	(103)
sypA	6.16 (±0.64)	Syringopeptin synthetase	(147)
sypB-1	7.78 (±0.74)	Syringopeptin synthetase	(147)
sypB-2	5.41 (±0.80)	Syringopeptin synthetase	(147)
sypC1	5.79 (±1.09)	Syringopeptin synthetase	(147)
sypC2	5.75 (±1.32)	Syringopeptin synthetase	(147)
ORF19	3.49 (±0.16)	Putative membrane protein	Kang and Gross, unpublished
sypD	6.23 (±0.59)	Putative ABC transporter	Kang and Gross, unpublished
dat	6.23 (±0.65)	Aminotransferase	Kang and Gross, unpublished
pseA	4.33 (±0.74)	Putative outer membrane protein	Kang and Gross, unpublished
pseB	1.56 (±0.15)	Efflux membrane fusion protein	Kang and Gross, unpublished
pseC	1.64 (±0.14)	Efflux membrane fusion protein	Kang and Gross, unpublished
ORF14	1.03 (±0.07)	Putative chemotaxis protein	Wang and Gross, unpublished
ORF13	0.91 (±0.08)	unknown	Wang and Gross, unpublished
ORF12	1.02 (±0.09)	Sensor protein	Wang and Gross, unpublished
ORF11	0.79 (±0.09)	Amino acid deaminase	Wang and Gross, unpublished
ORF10	1.30 (±0.16)	Hypothetical protein	Wang and Gross, unpublished
ORF9	1.20 (±0.11)	Hypothetical protein	Wang and Gross, unpublished
ORF8	1.17 (±0.10)	Hypothetical protein	Wang and Gross, unpublished
ORF7	1.06 (±0.14)	Hypothetical protein	Wang and Gross, unpublished
ORF6	0.84 (±0.19)	Hypothetical protein	Wang and Gross, unpublished
ORF5	0.88 (±0.08)	Hypothetical protein	Wang and Gross, unpublished
ORF4	1.25 (±0.45)	Hypothetical protein	Wang and Gross, unpublished
ORF3	0.75 (±0.08)	Hypothetical protein	Wang and Gross, unpublished
ORF2	1.27 (±0.17)	Hypothetical protein	Wang and Gross, unpublished
ORF1	1.03 (±0.10)	Hypothetical protein	Wang and Gross, unpublished
hrpL	2.93 (±0.44)	alternative sigma factor	(4)
hrpR	2.10 (±0.18)	Regulatory factor	(172)
hrpS	1.87 (±0.26)	Positive regulatory factor	(172)

TABLE 2.4. Continued

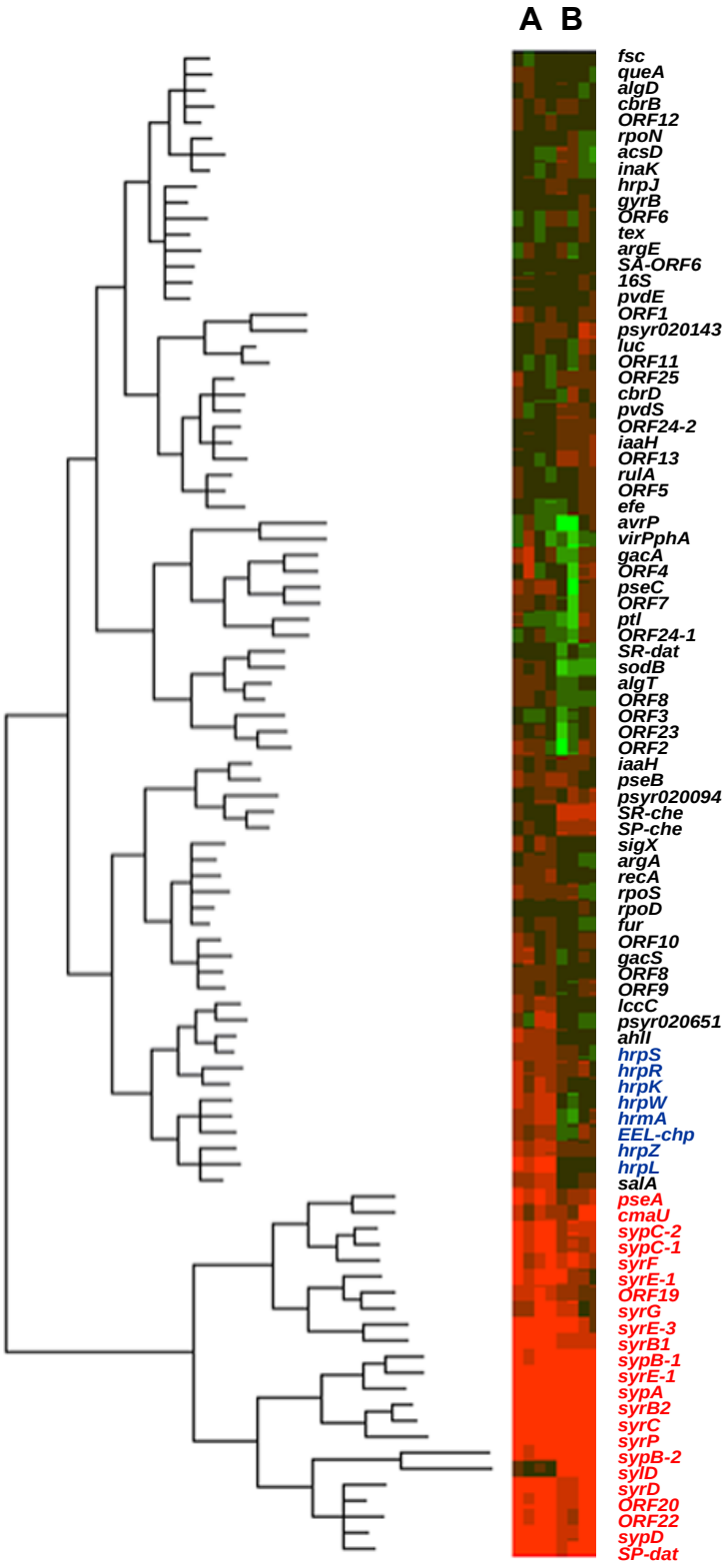
Genes	Ratio (S.E.M)	Gene product	References
hrpK	2.27 (±0.40)	Unknown	Wang and Gross, unpublished
hrpJ	1.01 (±0.16)	flagella biogenesis	(98)
hrpZ	3.95 (±0.28)	harpin	(68)
hrpW	2.63 (±0.23)	Type III effector	(26)
queA	1.02 (±0.11)	exchangeable effector locus	(4)
EEl-chp	2.0 (±0.28)	Type III chaperone protein	Wang and Gross, unpublished
hrmA	2.24 (±0.28)	Avr (effector) proteins	(4)
gacS	1.56 (±0.26)	sensor protein	(171)
gacA	1.57 (±0.35)	regulator protein	(136)
rpoS	1.41 (±0.08)	RNA polymerase sigma factor	(72)
rpoD	1.13 (±0.06)	Principle sigma factor	(159)
rpoN	0.82 (±0.06)	putative sigma-54 protein	(2)
sigX	1.37 (±0.23)	sigma factor	(21)
psyr020094	1.28 (±0.22)	Putative NRPS	www.jgi.doe.gov/JGI_microbial
psyr020651	1.86 (±0.14)	Putative NRPS	www.jgi.doe.gov/JGI_microbial
sylD	1.16 (±0.11)	Putative syringolin synthetase	(7)
algT	1.16 (±0.05)	Alternative sigma factor	(85)
algD	0.84 (±0.22)	GDP-mannose dehydrogenase	(47)
iaaM	1.21 (±0.14)	tryptophan monooxygenase	(108)
iaaH	0.84 (±0.07)	indoleacetamide hydrolase	(108)
ahlI	1.97 (±0.20)	acyl homoserine lactone synthetase	(87)
inaK	0.81 (±0.09)	Ice nucleation protein	(96)
pvdS	0.86 (±0.09)	Putative acetylase	(117)
pvdE	0.83 (±0.06)	pyoverdine synthetase	(110)
fur	1.19 (±0.14)	Ferric uptake regulator	(52)
lccC	2.09 (±0.66)	levansucrase	(94)
cbrB	1.03 (±0.13)	ferrisiderophore permease	(105)
cbrD	0.81 (±0.09)	ATP-binding unit in ABC transport	(105)
acsD	0.77 (±0.04)	Achromobactin biosynthetase	(52)
fsc	0.81 (±0.07)	ferric siderophore receptor	(120)
SR-dat	0.80 (±0.05)	Diaminobutyrate transaminase	(79)
psyr020143	1.02 (±0.19)	putative NRPS	www.jgi.doe.gov/JGI_microbial
tex	0.85 (±0.07)	S1 RNA binding domain protein	(120)
gshA	1.27 (±0.09)	Glutamate-cysteine ligase	(120)
argA	1.15 (±0.09)	N-acetylglutamate synthetase	(102)
argE	0.92 (±0.11)	Acetylornithine deacetylase	(102)
SA-ORF6	0.90 (±0.12)	unknown	(102)
rulA	0.85 (±0.09)	Radiation tolerance	(158)
sodB	1.11 (±0.05)	Iron-superoxide dismutase	(67)
gyrB	0.79 (±0.04)	DNA gyrase subunit B	(143)
recA	1.51 (±0.21)	Principal sigma factor	(90)
luc	0.86 (±0.10)	Luciferase	(35)
negc10	0.98 (±0.33)	Random primer	Qiagen
16S rDNA	1 (±0.0)	16S ribosomal RNA	(120)

* The *psyr* numbers stand for ORFs predicted by www.jgi.doe.gov/JGI_microbial.

Microarray analysis of the effects of plant signal molecules on expression of virulence-related genes

Analysis with a 70-mer oligonucleotide subgenomic microarray showed that 26 of 95 genes were induced significantly greater than two-fold by addition of arbutin (100 μM) and D-fructose (0.1%) (Fig. 2.1 and Table 2.4). In this study, no gene or ORF included was observed to have an expression ratio below 0.5, which indicates that no gene or ORF included in the microarray was negatively regulated by plant signal molecules. Genes dedicated to biosynthesis, secretion, and regulation of syringomycin and syringopeptin were induced by plant signal molecules. The expression of all of the syringopeptin synthetase genes, including *sypA*, *sypB*, and *sypC* (147) (Fig. 2.1 and Table 2.4), was increased 5.4- to 7.8-fold by addition of arbutin and D-fructose to SRM medium, respectively. The expression of all of the syringomycin biosynthesis genes, including *syrB1*, *syrB2*, *syrC*, and *syrE* genes (64, 101) (Fig. 2.1 and Table 2.4), was increased 4.4- to 10.5-fold by addition of plant signal molecules (Table 2.4). The expression levels observed from three oligonucleotides designed from different locations in *syrE* showed greater than 4-fold increases with the addition of arbutin and D-fructose. Three major regulatory genes located at the right border of the *syr-syp* genomic island, *salA*, *syrF*, and *syrG* were up-regulated by 2.5-, 3.9-, and 3.1-fold, respectively. Expression of most secretion genes in the *syr-syp* genomic island including *syrD*, *sypD*, two *mtrC* homologues, one *oprM* homologue, and *pseA*, was increased to higher levels of 2.5 to 6.5 times by plant signal molecules. In addition to the *syr-syp* genes, the *hrp* genes, *hrpZ*, *hrpR*, *hrpL*, *hrpW*, *hrmA*, and *hrpK* (4, 33) included on the microarray were

FIG. 2.4. Unsupervised hierarchical clustering analysis of the *syr-syp* and representative genes summarized in Table 2.4. Column A represents comparisons between B301D on SRM and SRM_{AF} agar media. Column B represents comparisons of gene expression between B301D and B301DSL7, a *salA* mutant. The scale of gene activities is represented from green (not induced or induced at low level) to red (induced at high level). The cluster analysis of microarray data was performed with the self-organizing tree algorithm (SOTA). The *syr-syp* genes that clustered together are in red. The genes associated with the Hrp Type III secretion system that are clustered together are in blue.


A B
fsc
queA
algD
cbrB
ORF12
rpoN
acsD
inaK
hrpJ
gyrB
ORF6
tex
argE
SA-ORF6
16S
pvdE
ORF1
psyr020143
luc
ORF11
ORF25
cbrD
pvdS
ORF24-2
iaaH
ORF13
rulA
ORF5
efe
avrP
virPphA
gacA
ORF4
pseC
ORF7
ptl
ORF24-1
SR-dat
sodB
algT
ORF8
ORF3
ORF23
ORF2
iaaH
pseB
psyr020094
SR-che
SP-che
sigX
argA
recA
rpoS
rpoD
fur
ORF10
gacS
ORF8
ORF9
lccC
psyr020651
ahlI
hrpS
hrpR
hrpK
hrpW
hrmA
EEL-chp
hrpZ
hrpL
salA
pseA
cmaU
sypC-2
sypC-1
syrF
syrE-1
ORF19
syrG
syrE-3
syrB1
sypB-1
syrE-1
sypA
syrB2
syrC
syrP
sypB-2
sylD
syrD
ORF20
ORF22
sypD
SP-dat

induced more than 2-fold in the presence of plant signal molecules (Table 2.4).

The availability of a transcriptional profile for B301D compared to B301DSL7 on PDA and that of B301D on SRM agar medium with or without exogenously added arbutin (100 μM) and fructose (0.1%) facilitated cluster analysis with the self organizing tree algorithm (SOTA) (74). As shown in Fig. 2.4, all of the induced genes were grouped into two clusters based on SOTA analysis, with the *syr-syp* genes clustered together and the *hrp*/*avr* genes forming a second cluster.

Housekeeping genes, such as *sigX* (21), *algT* (85), *algD* (47), *sodB* (67), and *inaK* (96) located outside of the *syr-syp* genomic island, were expressed at high levels on both SRM and SRM_{AF} media with no significant differences in expression levels between these two media. Genes involved in siderophore production [i.e., *pvdS* (117), *pvdE* (110), *fsc* (120), *acsD* (52), *cbrB* (105), *cbrD* (105), and *fur* (66)], environmental stress (*rulA*) (180), quorum sensing (*ahlI*) (87), global regulation [i.e., *gacS*, *gacA* (36), *rpoN* (2), *rpoS* (72), and *rpoD* (159)], phytohormone synthesis (*iaaM*, *iaaH*) (108), and alginate production (*algD*) (91) were not affected by plant signal molecules.

Independent microarray validation using GUS assays and quantitative real-rime transcription-PCR (QRT-PCR)

In addition to the microarray results of plant signal molecules on *syr-syp* genes, 10 genes (nine induced genes (i.e., *syrB1*, *syrC*, *sypA*, *sypB*, *hrpR*, *hrpZ*, *salA*, *syrF*, and *syrG*) and one unaffected gene (i.e., *recA*)) that represent a range of changes observed in microarray studies (0.8- to 10.5-fold changes) were validated by GUS assays and QRT-PCR analysis. GUS assays corroborated the direction of regulation observed from

microarray analysis for all three regulatory genes including *salA*, *syrF*, and *syrG* located at the right border of the *syr* gene cluster. Based on quantitative GUS assays, expression of pSL40F (*salA*::*uidA*), pSL38F (*syrF*::*uidA*), and pSL39F (*syrG*::*uidA*) was significantly increased by addition of arbutin and D-fructose. Strain B301D, carrying pSL40F, pSL38F, or pSL39F, demonstrated GUS activity of 1059, 1700, and 535 units per 10^8 CFU, respectively, 72 h after incubation at 25°C in SRM liquid medium. In contrast, their expression levels reached 1918, 2711, and 1220 units per 10^8 CFU, respectively, in SRM_{AF} liquid medium. Therefore, the expression levels of *salA*, *syrF*, and *syrG* were increased 181%, 159% and 224%, respectively, by addition of plant signal molecules.

The regulation patterns defined by QRT-PCR were similar to those determined by the microarray data. Based on QRT-PCR analysis, the fold induction by arbutin and fructose on *syrB1*, *syrC*, *sypA*, *sypB*, *hrpZ*, *hrpR,* and *recA* were 8.4, 16.3, 4.3, 25.7, 13.8, 6.0, and 1.2, respectively (Table 2.5). In comparison, the induction levels by microarray analysis were increased by 5.2-, 10.5-, 6.2-, 6.6-, 2.1-, 4.0-, and 1.5-fold, respectively (Table 2.4). The greatest difference in changes was obtained for *sypB*; the change of transcriptional level determined by QRT-PCR analysis was 25.7-fold, compared to a 6.6-fold change in expression by microarray analysis.

Induction of *syr-syp* and type III genes via different regulatory pathways

QRT-PCR was used to evaluate the effect of a *gacS* disruption on the induction of *syr-syp* and *hrp* genes. The *gacS* mutant (B301DG12) was inoculated on SRM and SRM_{AF} media for 3 days at 25°C as described above. In a *gacS* mutant, the

TABLE 2.5. Quantitative real-time PCR analysis of expression of representative genes of strains B301D and B301DG12 as influenced by the presence of plant signal molecules

Gene	Change in transcriptional level (± SE) on SRM versus SRM_{AF} [a]	
	B301D	B301DG12
syrB1	8.4 ± 0.7	1.4 ± 0.2
syrC	16.3 ± 1.6	1.8 ± 0.1
sypA	4.3 ± 0.1	1.0 ± 0.2
sypB	25.7 ± 2.0	3.4 ± 1.0
hrpR	13.7 ± 2.1	11.5 ± 2.7
hrpZ	6.0 ± 0.5	5.7 ± 0.4
recA	1.2 ± 0	1.0 ± 0

[a] Values represent the fold change in transcriptional level for strains grown on SRM_{AF} (100 μM arbutin, 0.1% D-fructose) as compared to growth on SRM medium. Each value represents the average of three independent assays followed by the standard error (SE) of the mean.

transcriptional expression increase of *sypA*, *sypB*, *syrB1*, and *syrC* caused by plant signal molecules was reduced to 1.0-, 3.4-, 1.4, and 1.8-fold as compared to 4.3-, 25.7-, 8.4-, and 16.3-fold increase by B301D, respectively (Table 2.5). There was no significant difference in the expression of *hrpR* and *hrpZ* between strains B301D and B301DG12 (Table 2.5). These data suggest that the plant signal transduction pathway responsible for toxin production is independent of that for Hrp Type III secretion system in B301D. In contrast, the expression of the housekeeping gene *recA* was not affected by the *gacS* mutation. The expression of the *recA* gene was comparable to that of B301D grown on SRM or SRM_{AF} media.

DISCUSSION

The induction of toxigenesis in *P. syringae* pv. *syringae* by specific plant signal molecules reflects an ability of the bacterium to adapt to a dynamic plant environment. It was reported that syringomycin production is activated by specific plant signal molecules in diverse strains of *P. syringae* pv. *syringae* (131). This study established that plant signal molecules likewise control expression of genes involved in syringopeptin production based on the following evidence. (i) Syringopeptin production by B3AR132 and BR132 are increased more than 1.6-fold by addition of arbutin and D-fructose to SRM agar medium as revealed by bioassays; (ii) GUS assays indicate that expression of *sypA*::*uidA* is increased substantially by addition of specific phenolic glycosides (i.e., arbutin, phenyl-β-D-glucopyranoside and salicin) to SRM liquid medium; (iii) Analysis with a subgenomic 70-mer oligonucleotide microarray demonstrates that the transcriptional levels of the *syr-syp* genes are activated 2.5- to

10.5-fold by arbutin and D-fructose; (iv) QRT-PCR analysis shows that the expression of the representative *syr-syp* genes are induced from 4.3- to 25.7-fold by arbutin and D-fructose. This is the first report that syringopeptin production and genes involved in syringopeptin production are activated by specific plant signal molecules.

Plant signal molecules have the same specificity for induction of the *syp* genes as that for the *syr* genes. Of the 10 phenolic compounds tested, only arbutin, salicin, and phenyl-β-D-glucopyranoside showed significant induction of expression of *sypA*::*uidA* in B301DSL29. As observed for *syrB1* (115), other representative phenolic compounds (hydroquinone, naringenin, phenol, phenyl-β-D-galactopyranoside, quercetin, rutin, and saligenin) did not affect the expression of *sypA*::*uidA*. In addition, specific sugars, such as D-fructose, enhanced phenolic glycoside-mediated induction of *sypA*, at levels similarly observed for *syrB1*. The similar specificity of induction of the *syrB1* and *sypA* synthetase genes by plant signal molecules is consistent with the fact that the SalA regulon (103) transduces the plant signals to the *syr-syp* genes. The efficient induction of *syrB1* and *sypA* genes by phenolic compounds and sugars resembles the process of *vir* gene activation in *A. tumefaciens* even though the active phenolic metabolites are different for the two bacteria (8, 24, 150). *A. tumefaciens* responds to acetosyringone (152) to induce the *vir* gene expression, while *P. syringae* pv. *syringae* responds to phenolic β-glycosides such as arbutin and salicin to activate the expression of syringomycin and syringopeptin associated genes. The phenolic β-glycosides are found in leaves, bark, and flowers of many plant species that are the host of *P. syringae* pv. *syringae* (115). Arbutin occurs in one monocot family, *Liliaceae*, and more than 10

dicot families, including *Rosaceae*, *Leguminosae*, and *Saxifragaceae*, which are all hosts of *P. syringae* pv. *syringae* (115). The phenolic compounds can occur in high concentrations in plant tissues. For example, pear (*Pyrus comunis*) leaves contain about 150mM arbutin (114). In addition, fructose and sucrose composed as much as 1% to 3% of the dry weight of the cherry tissues (86). It appears that plant signal molecules would be present in sufficient quantity to induce syringomycin and syringopeptin production by *P. syringae* pv. *syringae* in the invasion of plants as the substantial induction of *syrB*::*lacZ* (115) and *sypA*::*uidA* by arbutin (100 μM) and D-fructose (0.1%).

Microarray analysis is a powerful tool for monitoring expression of the *syr-syp* genes (103, 135). Analysis with 70-mer oligonucleotide subgenomic microarrays indicates that plant signal molecules induce the coordinated expression of the *syr-syp* genes. Once the effects of arbutin and D-fructose on syringomycin and syringopeptin production in B301D were established, microarray analysis was used to examine the signal activity of arbutin and D-fructose on expression of virulence-associated genes. It was clearly shown in the microarray analysis that not only are the toxin biosynthesis genes positively regulated, but regulatory and secretion genes associated with syringomycin and syringopeptin production are also induced by arbutin and D-fructose. The up-regulated *syr-syp* genes belong to the SalA regulon (103) as indicated by clustering analysis (Fig. 2.4), which demonstrates that the plant signal transduction of the *syr-syp* genes is mediated by SalA. It appears that cluster analysis is very useful in identifying specific pathways that are co-regulated based on their similar patterns of gene expression (19).

Besides the induction of syringomycin and syringopeptin, most *hrp* and *avr* genes included in this study were induced by the addition of arbutin and D-fructose. The induction of *hrp* and *avr* genes in plant-bacteria interactions was reported to be more common than previous realized (18, 133). Induction of *hrp* genes was studied mostly *in planta*, and the specific plant signal molecules involved in induction were not defined (133). *hrp* genes were shown to be expressed when bacteria are grown in defined minimal medium but not in complex media (10, 133, 167, 173). The SRM medium used in this study is different from the defined *hrp*-inducing medium and supports the expression of the *hrp* genes tested. The transcriptional levels of *hrpZ*, *hrpW*, *hrpL*, *hrpR*, and *hrpK* was increased more than two-fold in SRM_{AF} liquid medium, which demonstrates a link between plant signal molecules that induce phytotoxin and genes associated with the Hrp Type III secretion system.

Despite the fact that *syr-syp* and *hrp*/*avr* genes are activated by plant signal molecules, the two systems use different signal transduction pathways to induce virulence associated genes. The plant signal stimulon might involve the coordinated expression of numerous regulons, a situation similar to that of the heat shock stimulon (118, 176). As observed by cluster analysis (Fig. 2.4), the induced genes were grouped into the *syr-syp* and the *hrp*/*avr* clusters. The *syr-syp* genes were demonstrated to belong to the SalA regulon (103), while the *hrp*/*avr* genes were clustered together and belong to the HrpL regulon (51). In *P. syringae*, three regulatory genes, *hrpL*, *hrpR* and *hrpS*, are dedicated to *hrp/avr* gene expression (60, 172). *hrpR* and *hrpS* function as positive regulatory factors and encode enhancer-binding proteins which interact to activate *hrpL*

(Fig. 2.5) (78). Considerable research has been done to verify the relationship between *hrp* and GacS/GacA systems (27). GacS and GacA, which encode a two-component sensory transduction system in *P. syringae*, positively control *salA* (88). RT-PCR transcriptional analysis revealed that a *P. syringae gacS* mutation abolished induction of the four syringomycin/syringopeptin synthesis genes *syrB1*, *syrC*, *sypA* and *sypB* by plant signal molecules (Table 2.5). However, the induction of *hrpR* and *hrpZ* by plant signal molecules is not affected by mutation of *gacS*. This is consistent with the discovery that GacA is the master regulator of the *syr* and *hrp* genes in *P. syringae* pv. *tomato* DC3000, but it does not control *hrp* genes in *P. syringae* pv. *syringae* B728a (27). This demonstrated that the plant signal induction of syringomycin and syringopeptin in B301D is via the GacS pathway, while the induction of the *hrp* genes uses a different pathway.

Both the *salA* and *syrF* regulatory genes are required for syringopeptin production. It has been demonstrated that the regulation of syringopeptin and syringomycin is controlled by the same regulatory cascade. SalA is the key regulator in the control of syringomycin and syringopeptin production (101, 103). SyrF, which is controlled by SalA, positively regulates syringomycin production (101) and this study demonstrates that SyrF positively regulates syringopeptin production (Fig. 2.3). Standard phytotoxin bioassays showed that disruption of either *salA* or *syrF* resulted in the failure to produce syringopeptin on both SRM and SRM_{AF} agar media 4 days after inoculation (Fig. 2.3). Furthermore, the expression of *sypA*::*uidA* in either *salA* or *syrF*

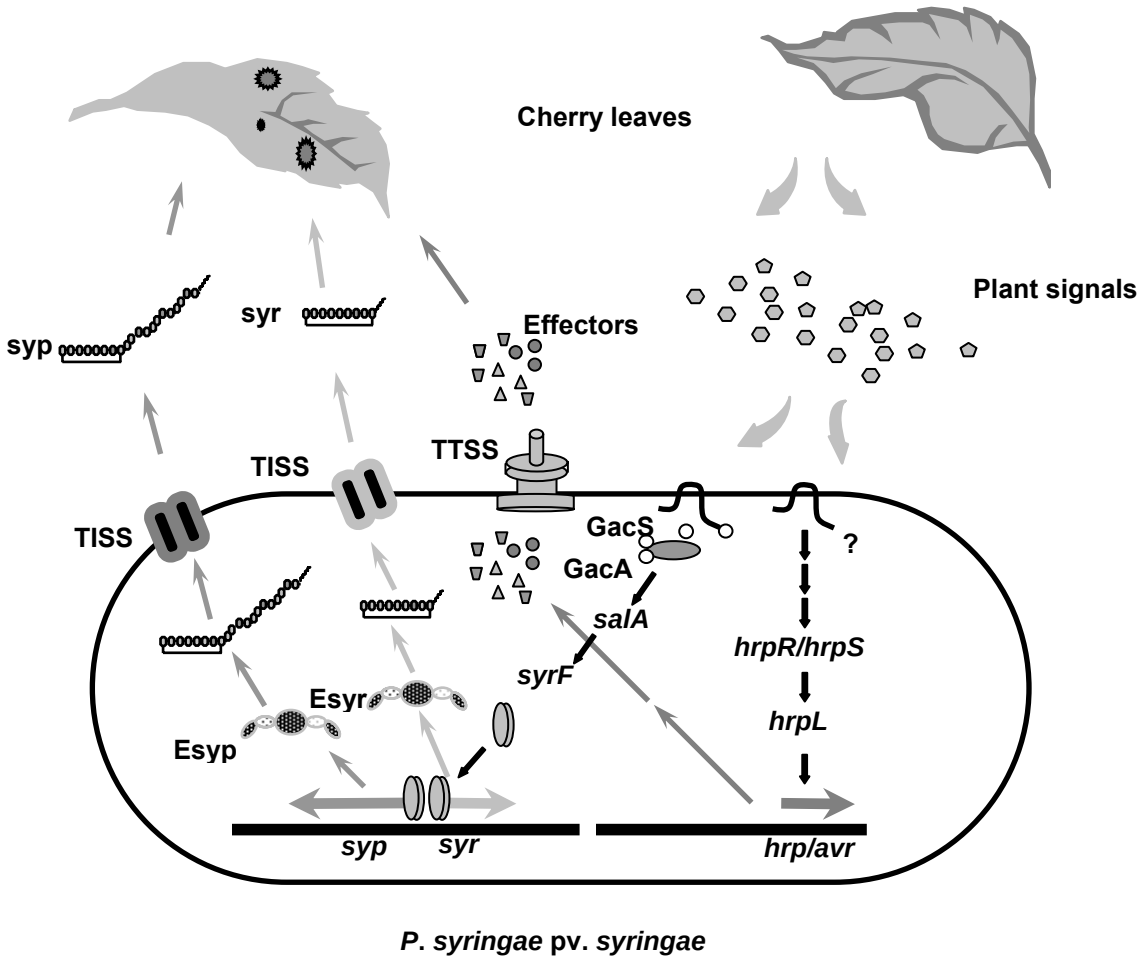


FIG. 2.5. A diagram of the stimulon induced by plant signal molecules in *P. syringae* pv. *syringae* B301D to activate phytotoxin production. Plant signal molecules in the plant apoplast activate the *gacS/gacA, salA, syrF* and *hrpL* signal transduction pathways leading to the transcription of the *syr-syp* toxin genes and genes associated with *hrp* Type III secretion. Strain B301D senses plant signals by GacS, a transmembrane sensor kinase, or some unknown acceptor senses the plant signals and transforms them to GacS. GacS then activates *salA* through the response regulator GacA. SalA transcriptionally activates expression of *syrF*, and SyrF in turn activates the *syr-syp* genes upon binding to the promoter region as a dimer. B301D also senses plant signal molecules through an unknown mechanism (represented as ?) to activate expression of the Hrp type III associated genes through the *hrpR*/*hrpS*/*hrpL* regulatory pathway. Expression of syringomycin (*syr*) and syringopeptin (*syp*) genes is induced by plant signal molecules, and the toxins are secreted through a Type I secretion system (TISS). The nonribosomal peptide synthetases for *syr* and *syp* are represented as Esyr and Esyp, respectively.

mutants was substantially decreased as compared to that of B301D grown in SRM and SRM_{AF} liquid media, respectively. Both SalA and SyrF belong to the LuxR regulatory protein family (82, 101). The LuxR protein contains a helix-turn-helix DNA-binding motif at the C terminus and regulates target genes by binding to the *lux* box in the promoter regions (43). It is very likely that SyrF controls the coordinated expression of the *syr-syp* genes by binding to the promoter regions directly.

Microarray analysis of the plant signal stimulon in *P. syringae* pv. *syringae* provides a valuable foundation for the study of the regulatory mechanism controlling syringomycin and syringopeptin production. Apparently, the bacterium coordinates the expression of the *syr-syp* and *hrp* genes in response to the plant environment (Fig. 2.5). As proposed in the model shown in Fig. 2.5, *P. syringae* pv. *syringae* senses plant signal molecules via the GacS/A pathway, which then activates the expression of the *syr-syp* genes through SalA and SyrF. It remains to be determined what genes are directly involved in the sensing of plant signal molecules and how SyrF controls syringomycin and syringopeptin production. It is expected that SyrF might control the coordinated expression of the *syr-syp* genes by directly binding to the promoters as suggested in the model proposed in Fig. 2.5. Currently, the promoter regions of the *syr-syp* genes and the functions of SyrF and SalA regulatory proteins are being characterized in an effort to understand the co-regulatory mechanism important for syringomycin and syringopeptin production.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media

The bacterial strains and plasmids used in this study are listed in Table 2.1. The *E. coli* strain DH10B (144) used for cloning was cultured at 37°C in Terrific Broth (TB) or on Luria-Bertani (LB) agar media. Strains of *P. syringae* pv. s*yringae* were routinely cultured at 25°C in nutrient broth-yeast extract (NBY) liquid or agar medium (164). To evaluate the effect of plant signal molecules on induction of syringopeptin production, syringomycin minimal SRM and SRM_{AF} (SRM medium containing 100 μM arbutin and 0.1% fructose) media were prepared as described by Mo and Gross (115). For tests of gene induction by plant signal molecules, *P. syringae* pv. s*yringae* was cultured in SRM (115) liquid or agar medium with or without plant signal molecules as identified below. Antibiotics (Sigma Chemical Co., St. Louis, MO) were added to media in the following concentrations: tetracycline 25 μg/ml, kanamycin 100 μg/ml, ampicillin 100 μg/ml, and gentamicin 5 μg/ml.

Bioassays for syringopeptin production

The *P. syringae* pv. *syringae* strains BR132 and B3AR132 were evaluated for syringopeptin production on SRM and SRM_{AF} media by using standard bioassays as previously described by Scholz-Schroeder et al. (146). Briefly, *P. syringae* pv. *syringae* strains were grown overnight in 2 ml of NBY liquid medium. Bacterial cells were harvested by centrifugation, washed once with sterile distilled water (SDW), and then resuspended in SDW to a concentration of approximately 2 x 10^8 CFU per ml. Aliquots (5 μl) of bacterial suspension were spotted onto SRM and SRM_{AF} plates. The strains

were cultured on plates for 4 days and then sprayed with *B. megaterium* Km and cultured overnight at 25°C. Because syringomycin inhibits the growth of *B. megaterium*, both strains tested for syringopeptin production were *syrB1* mutants, i.e., BR132 and B3AR132 (146). Zones of inhibition of *B. megaterium* were measured from the margins of bacterial colonies. Duplicate cultures were prepared for all treatments with all assays repeated three times on separate days.

Construction of GUS transcriptional fusions and procedures for GUS assays

The regulatory effects of *salA* and *syrF* genes on expression of the *sypA*::*uidA* gene fusion in cis were quantified by fluorometric analysis of GUS activity. Plasmid pSL19, which carries a disrupted *syrF* gene by insertion of the *nptII* cassette, was electroporated into the *sypA*::*uidA* reporter strain B301DSL29 (103) to generate the double mutant B301DNW31. The disrupted *syrF* gene in the double mutant was amplified by the polymerase chain reaction (PCR). Primers used in the amplification of *syrF* were PsyrFf: GCCATTCCTTGCGCCCATAAA and PsyrFr: CGAGGCAGAATTCCGACACAAG. The resulting PCR product from B301DNW31 was subcloned into the pGEM-T easy vector to generate plasmid pNWE32. The insertion of the *nptII* cassette into *syrF* was confirmed by sequencing using PsyrFf as the primer.

The effects of different plant signal molecules and regulatory genes (i.e., *salA*, *syrF*, and *syrG*) on expression of *syrB1::uidA* and *sypA::uidA* gene fusions were quantified by fluorometric tests of GUS activity. A modified fluorometric protocol was used for analysis of GUS activity (101). Bacterial cells were cultured with

shaking in 2 ml of NBY broth overnight at 25°C, harvested by centrifugation, washed twice with SDW and then diluted with 20% glycerol to an optical density of 0.6 at 600 nm. Stock cell suspensions were stored at -80°C. Cell stock suspensions (50 µl) were used to inoculate 4 ml of PDB medium or SRM liquid medium with or without plant signal molecules and incubated for 72 h at 25°C without shaking. Cell cultures (50 µl) were collected by centrifugation, resuspended in 100 µl of GUS extract buffer (GEB) (173), and lysed by sonication. Duplicate cultures were prepared for GUS assays with all assays repeated three times on separate days.

Phenolic compounds, sugars, and organic acids tested for *sypA*-inducing activity

Phenolic compounds tested for plant signal activity were arbutin (ICN Biomedicals Inc., Aurora, OH), hydroquinone, naringenin, phenol, phenyl-β-D-glucopyranoside, phenyl-β-D-galactopyranoside, quercetin, rutin, salicin, and saligenin (Sigma Chemical Co., St. Louis, MO). D-fructose, which is known to enhance the signal activities of phenolic compounds (115), was purchased from Baker Chem. Co. (Phillipsburg, NJ).

Microarray analysis

To test the effects of arbutin and D-fructose on the expression of *syr-syp* genes and genes involved in production of factors associated with plant pathogenesis, microarray analysis was performed as described by Lu et al. (103). Strains of *P. syringae* pv. *syringae* were cultured with shaking at 25°C overnight in NBY medium (2 ml). Cells were harvested by centrifugation, washed twice, and then diluted with SDW to a concentration of approximately 2 x 10^8 CFU per ml. Cell suspensions (50 µl) were

spread on SRM and SRM_{AF} plates. The inoculated plates were incubated at 25°C for 72 h prior to recovery of cells. Total RNA was purified using a RiboPure-Bacteria kit (Ambion, Inc., Austin, TX). Total RNA (50 µg) was labeled with either Cy3-dUTP or Cy5-dUTP as described previously (103). Glass DNA microarrays containing a set of 70mer oligonucleotides, designed and synthesized by Qiagen, were produced to represent genes contained in the *syr-syp* genomic island and other genes associated with virulence. The microarrays were used to quantify relative mRNA levels by parallel two-color hybridization according to protocols described in detail elsewhere (103). Briefly, hybridization was performed at 60°C overnight in a moist chamber. After washing, the slides were dried by centrifugation and scanned immediately using a GenePix 4000b scanner (Axon Instruments Inc., Foster City, CA) to visualize the hybridization images.

Signal intensity and ratios were generated using GenePix Pro software provided with the scanner. Microarray data with intensities reproducibly higher than that of the background level were selected for analysis. The raw data was normalized using 16S rRNA as a standard. Hybridization experiments were conducted three times and each slide contained duplicate arrays.

Hierarchical clustering

Unsupervised hierarchical clustering analysis of *syr-syp* and relevant genes were performed with the self-organizing tree algorithm (SOTA) (http://www.almabioinfo.com/sota/). Cluster analysis was visualized with Treeview software (134).

QRT-PCR analysis

The effect of arbutin and fructose on *syrC*, *sypB*, *hrpR*, *hrpZ*, and *inaK* observed in microarray analysis was evaluated by quantitative real-time PCR using the QuantiTect SYBR Green RT-PCR kit (Qiagen Inc., Valencia, CA). QRT-PCR also was used to assess the effect of disruption of the *gacS* global regulatory gene on expression of the *syrC*, *sypB*, *hrpR*, and *hrpZ* genes. Primers used for RT-PCR were designed using the Lasergene Expert Sequence Analysis Package (DNAstar, Madison, WI) and are listed in Table 2.6. Consistent with microarray analysis, primers specific for the 16S rRNA gene were used for normalization controls. The linearity of detection of each primer pair was confirmed to have a correlation coefficient of at least 0.98 (r^2>0.98) over the detection area by measuring a five-fold dilution curve with total RNA isolated from bacterial cells. Reverse transcription was conducted at 50°C for 30 min with 100 ng total RNA as template, then followed by initial activation of HotStarTaq DNA Polymerase (95°C, 15 min). Totally, 30 cycles of RT-PCR reactions (94°C for 15 s; 55°C for 30 s, and 72°C for 30 s) were performed followed by melting curve analysis.

TABLE 2.6. Primers used for quantitative real-time PCR analysis

Name	Sequence	Resource
sypAF	TGCGGGTCGAGGCGTTTTTG	(83)
sypAR	GTTGCCGCGTCCTTGTCTGA	(83)
sypBF	TTCGATCAGGGTCACCGCCAACAATG	(103)
sypBR	AGCTGCTCAATGTCGAAAAGGTC	(103)
syrB1F	TTAGCGCCGCGTCAGCCCCTCTCAAG	(83)
syrB1R	GCTCAACGTCCGGGCTGCATCGCTCA	(83)
syrCF	ACCTGCAAGCGATGTTCCTC	(103)
syrCR	TGCCAGCTCGGTCTTGTTCA	(103)
hrpRF	TTCGGCGTGGTCAATGGTGCGTTCA	This study
hrpRR	CGTGTCGATTTCGTCCAGATAGAGG	This study
hrpZF	TCCTGAAACCGAGACGACTGG	This study
hrpZR	GACCGTTGCGCATCAGTTCCTC	This study
recAF	CTTCGGTACGCCTGGACA	(103)
recAR	AACTCGGCCTGACGGAAC	(103)
16SF	ACACCGCCCGT CACACCA	(103)
16SR	GTTCCCCTACGGCTACCTT	(103)

CHAPTER III

IDENTIFICATION OF THE *syr-syp* BOX IN THE PROMOTER REGIONS OF THE *syr-syp* GENES DEDICATED TO SYRINGOMYCIN AND SYRINGOPEPTIN PRODUCTION BY *P. syringae* pv. *syringae*

OVERVIEW

The phytotoxins syringopeptin and syringomycin are synthesized by nonribosomal peptide synthetases which are encoded by the syringomycin (*syr*) and syringopeptin (*syp*) genomic island in *P. syringae* pv. *syringae*. Previous studies demonstrated that expression of the *syr-syp* genes was controlled by the *salA*/*syrF* regulatory pathway, and induced by plant signal molecules. In this study, the 132-kb *syr-syp* genomic island was found to be organized into five operons along with and seven individual genes based on reverse transcriptional PCR (RT-PCR) and computer analysis. The transcriptional start sites of the *salA* gene and operons III and IV and the *salA* gene were located 63, 75, 104, and 10463-bp upstream of the start codons of *salA*, *syrP*, and *syrB1*, and *salA*, respectively, using primer extension analysis. The predicted -10/-35 promoter region of operon IV was confirmed based on deletion and site-directed mutagenesis analyses of the *syrB1::uidA* reporter with β-Glucuronidase (GUS) assays. A 20-bp conserved sequence (TGTCccgN4cggGACA, termed the *syr-syp* box) with dyad symmetry around the -35 region was identified via computer analysis for the *syr-syp* genes/operons responsible for biosynthesis and secretion of syringomycin and syringopeptin. Expression of the *syrB1*::*uidA* fusion was decreased 59% when 6-bp was

deleted from the 5' end of the *syr-syp* box in the promoter region of operon IV. These results demonstrate that the conserved promoter sequences of the *syr-syp* genes contribute to the co-regulation of syringomycin and syringopeptin production.

INTRODUCTION

Bacteria coordinate expression of functionally related genes to adapt to a rapidly changing environment (161). For example, enzymes that belong to the same metabolic pathway are always upregulated and downregulated together (161). Gene expression is most frequently modulated at the transcriptional level by the interaction of transcriptional factors and promoters in bacteria (156). Syringomycin (syr) and syringopeptin (syp) are lipodepsipeptide toxins produced by *P. syringae* pv. *syringae* through a nonribosomal peptide synthetase system (15). The *syr* and *syp* gene clusters dedicated to syringomycin and syringopeptin production are located adjacent to one another on the chromosome (Fig. 3.1) (147). The *syr-syp* genes are subjected to coordinated control by SalA and SyrF in response to environmental signals (see Chapter I). Similarly, coronatine biosynthesis genes are co-regulated (125, 166). Coronatine is a chlorosis-inducing phytotoxin produced by several pathovars of *P. syringae* and consists of two distinct moieties, a cyclized amino acid (coronamic acid, CMA) and a polyketide component (coronafacic acid, CFA). CMA and CFA are derived from separate biosynthesis pathways and linked via amide bond formation (13). The CMA biosynthetic gene cluster contains *cmaA*, *cmaB*, *cmaT*, and *cmaU* and the CFA gene

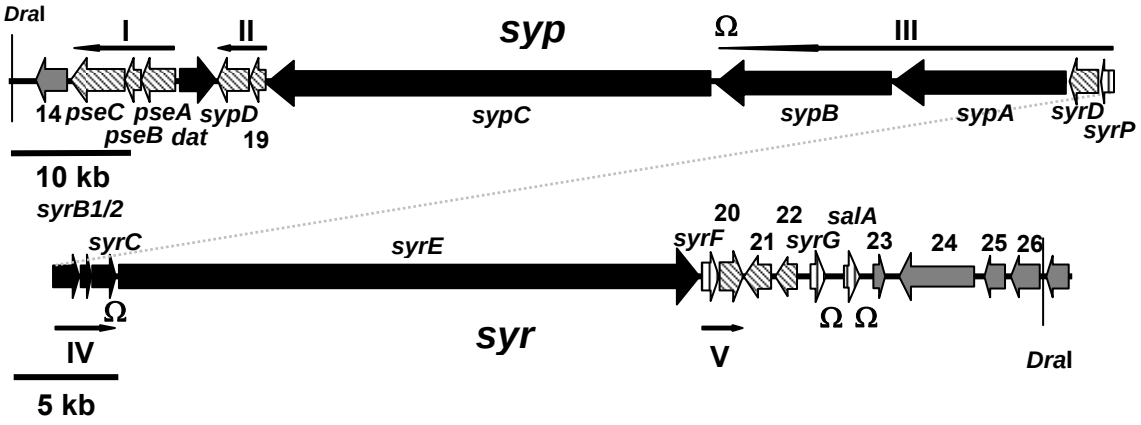


FIG. 3.1. Operon analysis of a 132-kb genomic DNA region of *P. syringae* pv. *syringae* strain B301D containing both syringomycin (*syr*) and syringopeptin (*syp*) gene clusters. The positions and orientations of the known and potential open reading frames (ORFs) are shown as horizontal arrows. The solid, diagonally-striped, and vertically-striped arrows represent genes that are predicted to be involved, respectively, in the synthesis, regulation, and secretion of the phytotoxins. The gray arrows represent the potential ORFs for which functions remain unknown. Operons I to V are indicated by thin black arrows. Putative rho-independent terminators are represented by Ω.

cluster consists of 10ten co-transcribed genes designated *cfl* and *cfa1*-9 (14). The CMA and CFA gene clusters are co-regulated by transcriptional activator CorR, which binds to both *cmaABT* and *cfl*/CFA promoter regions, respectively (125, 166). The PhoP/PhoQ two-component regulatory system controls the expression of more than 40 genes associated with virulence and fitness in *Salmonella enterica* (119). Similarly, the *syr-syp* genes are subjected to coordinated control by SalA and SyrF in response to environmental signals. Apparently, co-regulation is widely used in bacteria to maximize efficiency in utilizing resources and to enhance its competitiveness in the environment.

Generally, genes are transcriptionally co-regulated because they share high similarity in their promoter regions so that the genes are controlled by the same sigma factors or regulators (32). For example, the *hrp* box has been identified for most *hrp* and type III effector genes controlled by the alternative sigma factor HrpL (51). A conserved sequence (TnrA box) has been identified in the promoters of 17 TnrA-regulated genes in *Bacillus subtilis* (175). In *E. coli,* LexA was reported to regulate 31 genes by binding to a consensus sequence of TACTG(TA)5CAGTA in the promoter regions of the target genes (49). This demonstrates that the conservation of the promoter regions is vital to the coordinated expression of the genes in one regulon.

The sigma subunit of RNA polymerase is the main transcriptional factor, which is responsible for binding specificity for -10/-35 regions and determining the initiation of transcription in prokaryotes (71). The majority of sigma factors belong to the sigma 70 family. Multiple alternative sigma factors have been identified as a means of switching on specific regulons (100). For example, approximately 63 alternative sigma factors

were identified in the bacterium *Streptomyces coelicolor* (16). Among them, sigma 38 is the second major sigma factor and it mainly functions in stationary phase as well as several different stress conditions (72, 163). Members of the sigma 70 family recognize the -10 and -35 regions upstream of the transcriptional start site (122). In contrast, the sigma 54 family, a second family of sigma factors, recognizes sequences including short elements at nucleotides -12 and -24 (23) with extensive conservation between the two elements (11). Consequently, the promoter region is the key subject for transcriptional regulation in prokaryotes.

Previous studies demonstrated that genes dedicated to biosynthesis, secretion, and regulation of syringomycin and syringopeptin production by *P. syringae* pv. *syringae* are coordinately regulated at the transcriptional level (103). Transcriptional analysis with 70-mer oligonucleotide microarrays, along with GUS assays and real-time PCR (RT-PCR) analysis, demonstrated that all of the *syr-syp* genes (Fig. 3.1) belong to the SalA regulon (103). All the *syr-syp* genes belong to and the stimulon of plant signal molecules (see Chapter I). It was revealed that expression of the *syr-syp* genes were significantly higher in B301D than in the *salA* mutant (103) and were activated by arbutin (100μM) and D-fructose (0.1%) (see Chapter II). Genes activated include, including synthetase genes for syringomycin (i.e., *syrB1* and *syrE*) and syringopeptin (i.e., *sypA*, *sypB*, and *sypC*), four regulatory genes (i.e., *salA*, *syrF*, *syrG* and *syrP*), and nine putative secretion genes (i.e., *syrD*, *syrP*, *pseA*, *pseB*, *pseC*, two *mtrC* homologs, one *oprM* homolog, and one ORF encoding putative threonine efflux protein) (Fig. 3.1), dedicated to production of the two toxins (103). Furthermore, study indicates the all of

the *syr-syp* genes responsible for biosynthesis and secretion of syringomycin and syringopeptin belong to the SyrF regulon (Wang and Gross, unpublished data). Both SalA and SyrF belong to the LuxR regulatory protein family (42, 101). The LuxR protein contains a helix-turn-helix DNA-binding motif at the C terminus and regulates target genes by binding to the *lux* box. The *lux* box is 20-bp in length with a dyad symmetry centered at the -42.5 position, relative to the transcriptional start site of *luxI* (43). It was predicted that similar sequences are present in the *syr-syp* promoters region and are responsible for the co-regulation of syringomycin and syringopeptin production.

Operons are the principal form of gene organization and regulation in prokaryotes (92). For example, as reported for the 933 *E. coli* genes in the Regulon DB database, 124 genes are transcribed as single units, whereas the others are clustered into 237 operons (77, 141). Further study indicates the whole genome of *E. coli*, about 54% of all of the 4300 protein-coding genes (17), are organized into approximately 700 operons (140). In an operon, multiple genes are transcribed together from the same promoter into a single mRNA molecule (46). The organization of genes into operons provides the advantage of coordinated regulation of functionally related genes.

Despite progress made in understanding the regulation of syringomycin and syringopeptin production (101, 103), the transcription pattern of the *syr-syp* genes remains largely unknown. In addition, the promoters of the *syr-syp* genes have not been studied in depth. The objectives of this study were to carry out a detailed transcriptional analysis of the *syr-syp* genes, including characterizing the operon structure, the transcriptional start sites, and the common characteristics of the promoter regions of the

TABLE 3.1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Source
Escherichia coli		
DH10B	F⁻ *mcrA* Δ*lacX74* (ϕ80d*lacZ*ΔM15) Δ(mrr-*hsdRMS-mcrB*) *deoR recA1 endA1 araD139* Δ(*ara, leu*)7697 *galU galK* λ⁻ *rpsL nupG*	(57)
P. syringae pv. *syringae*		
B301D	Wild type, from pear	(31)
B301DNW201	*rpoS*::*nptII* derivative of B301D; Km^r	This study
Plasmid		
pBluescriptSK(+)	Cloning vector; Ap^r	Strategene. La Jolla, CA
pUCP26	Cloning vector; Tc^r Ap^r	Olsen et al. 1982
pBI101	Binary vector containing *uidA* gene; Km^r	Clonetech, Palo Alta, CA
pBSL15	Kanamycin resistance gene cassette; Km^r	(3)
pUCGM	Vector containing gentamycin resistance gene cassette; Gm^r	(149)
p601D-1	pRK415 carrying the 8-kb *Hin*dIII fragment of pYM101 with deletion of *Eco*RV fragment upstream of *syrB1*; Tc^r	Zhang and Gross, unpublished data
pSL2	pBI101 with the 0.85-kb aacC1 gene of pUCGM inserted at the *Eco*RI site downstream of the *uidA* gene; Gm^r Km^r	(101)
pSL8	pBR325 carrying the 3.0-kb *Eco*RI fragment of p29 containing *salA*, Tc^r Ap^r	(101)
pSL103	pUC18 carring a 8-kb *Eco*RI-*Kpn*I fragment from pBS008 containing *sypA*::*uidA-aaCC1* at BstZ17I partial *syrD* and start of *sypA*, Ap^r	(103)
pBS008	pUC18 carrying a 8-kb *Eco*RI-*Hin*dIII fragment from BS008 with *sypA*::*uidA-aaCC1* insertion at *Bst*Z17I site of *sypA*, Ap^r Gm^r	(146)
pYM101	pUC19 carring a 16-kb *Hin*dIII DNA fragment from pYM1 containing 5' end of *syrE*, all *syrC*, *syrB1*, *syrB2*, *syrD*, *syrP*, and 3' end of *sypA*, Ap^r	(132)
pSLB4	pUC18 carrying 5.0-kb fragment of p601D-1 with the *uidA-aacC1* fragment from pSL2 in-frame of *syrB1* at the *Eag*I site in forward orientation; Ap^r Gm^r	S.E.Lu and D.C. Gross, unpublished data
pGEMTrpos	pGEM-Teasy vector carrying a 3.1-kb fragment containing amplified *rpoS* of B301D; Ap^r	This study
pGEMTrposKm	pGEM-Teasy vector carrying a 4.3-kb fragment containing amplified *rpoS* of B301D with *nptII* insertion at the *Cla*I site of *rpoS*; Ap^r Km^r	This study
pBR325rposKm	pBR325 vector carrying a 4.3-kb fragment containing amplified *rpoS* of B301D with *nptII* insertion at the *Cla*I site of *rpoS*; Tc^r Km^r	This study
pSL105	pUCP26 carrying a 8-kb *Eco*RI-*Hin*dIII fragment from BS008 with *sypA*::*uidA-aaCC1* insertion at *Bst*Z17I site of *sypA*, Tc^r Gm^r	Lu and Gross, unpublished data
pNW105	pUCP26 carrying *sypA*::*uidA-aaCC1* and the upstream DNA sequence containg *syrD*, *syrP*, *syrB1*, *syrB2* and 5' end of *syrC*, Tc^r Gm^r	This study
pNW105-1	pUCP26 carrying *sypA*::*uidA-aaCC1*, *syrD*, *syrP* and 335-bp upstream the start codon of *syrP*, Tc^r Gm^r	This study
pNW105-2	pUCP26 carrying *sypA*::*uidA-aaCC1*, *syrD*, *syrP* and 59-bp upstream the start codon of *syrP*, Tc^r Gm^r	This study
pNW105-3	pUCP26 carrying *sypA*::*uidA-aaCC1*, *syrD*, 3' end of *syrP* (500-bp downstream the start codon of *syrP*, Tc^r Gm^r	This study
pNW104	pUCP26 carrying *Hin*dIII-*Eco*R fragment from pSLB4 containing the 3.2-kb *uidA-aacC1* fragment from pSL2 in-frame of *syrB1* at the *Eag*I site in forward orientation; Tc^r Gm^r	This study
pNW104-1	pUCP26 carrying *syrB1*::*uidA-aaCC1* and 391-bp upstream the start codon of *syrB1*, Tc^r Gm^r	This study
pNW104-2	pUCP26 carrying *syrB1*::*uidA-aaCC1* and 276-bp upstream the start codon of *syrB1*, Tc^r Gm^r	This study
pNW104-3	pUCP26 carrying *syrB1*::*uidA-aaCC1* and 218-bp upstream the start codon of *syrB1*, Tc^r Gm^r	This study
pNW104-4	pUCP26 carrying *syrB1*::*uidA-aaCC1* and 180-bp upstream the start codon of *syrB1*, Tc^r Gm^r	This study
pNW104-5	pUCP26 carrying *syrB1*::*uidA-aaCC1* and 111-bp upstream of the start codon of *syrB1*, Tc^r Gm^r	This study
pNW104-6	pUCP26 carrying *syrB1*::*uidA-aaCC1* and 218-bp upstream the start codon of *syrB1* with the potential -10 region was substituted with CTGCAG, Tc^r Gm^r	This study
pNW104-7	pUCP26 carrying *syrB1*::*uidA-aaCC1* and 218-bp upstream the start codon of *syrB1* with the potential -35 region was substituted with CTGCAG, Tc^r Gm^r	This study
pNW104-8	pUCP26 carrying *syrB1*::*uidA-aaCC1* and 146-bp upstream the start codon of *syrB1*, Tc^r Gm^r	This study
pNW104-9	pUCP26 carrying *syrB1*::*uidA-aaCC1* and 218-bp upstream the start codon of *syrB1* with TGTCCC of the *syr-syp* box was substituted with CTGCAG, Tc^r Gm^r	This study

TABLE 3.2. Primers used for reverse transcriptional PCR analysis, PCR, and site-directed mutagenesis

Name	Sequence	Resource
datRTF	CAAGGGATCGACTATGGAAGA	This study
sypDRTF	ATGTCGAAATGCCAGCTATCTACG	This study
pseCRTF	GCCATTAACGCCGGATCAAGGTCA	This study
chemRTF	ATACCGAGCATCCCCAAACCAC	This study
pseBRTR	TCGCGGTGGATGACGGGATTAT	This study
pseBRTF	AACATTTCACTGCCCGCCGATACCACAT	This study
pseARTR	TTCGGGAAATACCTGCCAACCTGT	This study
sypAPER1	TTCTTCTCGCAAAGGGGC	This study
sypAPER2	CTCCAGATGGCCGCCGATGTTGTA	This study
syrPR6	ACGCCCGTGATCCTTATGAA	This study
syrB1RTF	ATGCGTCCCTCGGTGTATGTG	This study
syrB1RTR	GGCTGGCCAGGAAATCATCGTC	This study
sypBRTR	TTCCCGGCTGGCAATCGTGA	This study
sypARTF	GGCCCATCGGAAACCACTACCTACTCG	This study
sypBRTF	CGGACCCGTTCAGCCTCACA	This study
sypCRTR	TGAGCCAGTCGCCGCCCATTC	This study
syrB1RTR2	ATCGGCCTCATCAAGACCAC	This study
syrPR2	TGCTCGCAGAAGAACCATTG	This study
syrFRTF	TGAACGCCCGGATGAAAAACCAG	This study
oprMRTR	GTCGGGTCGCGGAACAGGTCACG	This study
syrFRTR	TCTTGCAGCGAGTAATCCTTGTCAGC	This study
syrCRTF	TGAATGACGCGCTGAACAAGAC	This study
syrERTR	AGCTTGAACGGCGCCTTGGACAGA	This study
syrPRTF	CGGTGCATCTGCTCGGTCATTC	This study
sypCRTR1	CCAGGTGCGGCAACAGGTGATA	This study
mtrCspRTF	GTTGTTGGTTCTGGCAGCGGGTAT	This study
syrCRTR1	TCAAGGCGTCGAACCAGTC	This study
syrB2RTF	AAGAGTTCGGCGGCACCATTA	This study
mtrCspRTR	AATGGCTTTGGCGTCCTTGAG	This study
syrPRTF	CTTTACAGCCGCACCTTCA	This study
syrDRTR2	AACACCGCCGACTCCACCA	This study
syrDRTF	GTTTTGCCCTGGGACCTATCG	This study
syrARTR	GGGCGTAAATCATGCAGTAGAAGC	This study
salAPE	GTTGCATGTTCATCGGGGTTCCTT	This study
syrB1PE	GCATGACCAAAGCTCCTGTGTAAT	This study
syrPPE	GACCTCAGCCCTTCACATCCACTT	This study
syrERTF	CCCATATCGCCATCGGTAACTA	This study
sypAPE	GGCAGCCGGTAGTCAGGTCGTG	This study
mtrCR	CTATCAGCCGCAGCAGAAGTTAC	This study
cmaURTF	CAGCCCTTTCGGATTGTTGAGTG	This study
syrCRTR2	TCTCGATGTGCGGCACTGCACC	This study
syrBR11	ATGAGAATTCGCATGACCAAAGCTCCTGTGTA	This study
syrPR15	ATGAGAATTCCGCGTACCTGCCGAAAGAG	This study
syrBR24	ATCTGAATTCGTGCTGGTCTGGCGCCCTACAAAA	This study
B8SDM1F	TGATGGCCTAAGGCGTCTGAAGCTTCAATCCGGGACATCGGTCG	This study
B8SDM1R	CGACCGATGTCCCGGATTGAAGCTTCAGACGCCTTAGGCCATCA	This study
B8SDM2F	CGGTCGCGAAGAGTGTCGAAAGCTTCTGATCTGAATCGGCAGGC	This study
B8SDM2R	CCTGCCGATTCAGATCAGAAGCTTTCGACACTCTTCGCGACCG	This study
B8SDM3F	GCCCGTTAGTCGGTGCCTGCAGGCGCCAACTGCGGATC	This study
B8SDM3R	GATCCGCAGTTGGCGCCTGCAGGCACCGACTAACGGGC	This study
B8SDM4F	TTGTCCCCATTTCCCTCCTGCAGAGCCCGTTAGTCGGTGC	This study
B8SDM4R	GCACCGACTAACGGGCTCTGCAGGAGGGAAATGGGGACAA	This study
B8SDM5F	CTGAATCGGCAGGCTTCTGCAGCATTTCCCTCGACAGA	This study
B8SDM5R	TCTGTCGAGGGAAATGCTGCAGAAGCCTGCCGATTCAG	This study
B8SDM6F	CGGCAGGCTTTGTCCCCAAGCTTCTCGACAGACGCAGCCC	This study
B8SDM6R	GGGCTGCGTCTGTCGAGAAGCTTGGGGACAAAGCCTGCCG	This study
syrDF7	CAGAGCCGTTCGATGTAGTTGT	This study
rpoSR1	TCGCGTGCTTGAGACTGT	This study
rpoSF1	ACGCTTGAGCCTGTTCCTACT	This study

syr-syp genomic island. Efforts were also made to identify potential sigma factors that are associated with the promoter regions of the *syr-syp* genes.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media

The bacterial strains and plasmids used in this study are listed in Table 3.1. *E. coli* strain DH10B (57) was used for cloning and was cultured at 37°C in Terrific Broth (TB) (160) or on Luria Broth (LB) agar (142). *P. syringae* pv. s*yringae* strains were routinely cultured at 25°C in nutrient broth-yeast extract (NBY) broth or on NBY agar medium (164). For GUS assay experiments, *P. syringae* pv. s*yringae* strains were cultured in potato-dextrose broth (PDB) medium (61). Antibiotics (Sigma Chemical Co., St. Louis, MO) were added to media in the following concentrations: 25 µg of tetracycline per ml, 100 µg of kanamycin per ml, 100 µg of ampicillin per ml and 5 µg of gentamycin per ml.

Operon analysis of the *syr-syp* genomic island using RT-PCR

RT-PCR analysis was performed to define the transcripts for the *syr-syp* genes. For each pair of neighboring genes, specific primers (Table 3.2) were used to identify the putative transcript. For convenience, the RT-PCR products were named after the genes or ORFs at the two ends of transcript. Total RNA was prepared from strain B301D cells after 3 days of incubation on PDA at 25°C for 72 h using the RNeasy Mini Kit (Qiagen Inc., Valencia, CA) according to the method described in the manufacturer's directions. RNA was treated with RNase-Free DNase (Qiagen Inc., Valencia, CA) during isolation. Total RNA (0.1 µg) from strain B301D was used as a

template, and the primers used for the RT-PCR analysis are listed in Table 3.2. RT-PCR was performed with a One Step RT-PCR Kit (Qiagen Inc., Valencia, CA). After the reverse transcription reaction at 50°C for 30 min, PCR was carried out using the following conditions: 95°C for 15 min, 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 1 to 2 min (variable according to the length of the predicted products). For negative controls, template RNA was not added to the reaction mixtures. The RT-PCR products were subjected to electrophoresis with a 1.0% agrose gel. When no RT-PCR product was obtained with RNA as template, genomic DNA was used to test the fidelity of the primer pairs.

Primer extension analysis

Primer extension was performed with the Primer Extension System (Promega Corporation Madison, WI) and the sequence marker was generated with the SequiTherm EXCEL^{TM} II DNA Sequencing Kit (EPICENTRE, Madison, WI) according to the manufacturer's instructions. Oligonucleotides syrPPE, syrB1PE, and salAPE were 5' end-labeled with gamma-^{32}P ATP (Perkin-Elmer Life Sciences, Inc. Boston, MA). The primer extension reaction was done with 1.0 pmol of labeled primer and 15 µg of total RNA from strain B301D prepared as described above. The sequence ladders for the upstream regions of *syrP* and *syrB1* were obtained with pYM101 (131) as a template. Plasmid pSL008 (101) was used as a template for generating the *salA* sequence ladder.

Computer analysis

A program was designed to search for conserved sequences in the promoter regions of the *syp-syp* genes and operons controlled by *salA*/*syrF*. For an imperfect dyad

symmetry, two end elements are separated by a short arbitrary sequence with variable lengths (length is zero for a perfect dyad symmetry). A combinatorial (l, d, w)-dyad symmetry model, where l is the length of an element, d is the maximal number of mismatches allowed between two corresponding elements, and w is the length of the arbitrary sequence, was used. We used l=6, d=1, and w=11 in this study. Imperfect dyad symmetries were sought by identifying motifs that consist of two parts w1 and w2, each of length l, so that w1 and w2 are almost perfect inverted repeats of each other and they are separated by a short region of variable lengths and content. The resultant sequences were aligned with T-Coffee (121).

Construction of GUS transcriptional fusions

To identify the promoter region of operon IV, 5' *cis* promoter deletion constructs were made from pNW104, a transcriptional fusion construct containing the *syrB1* gene with a promoterless *uidA* gene insertion in the *Eag*I site of *syrB1*. To construct pNW104, the *Eco*RI-*Hin*dIII fragment containing *syrB1*::*uidA-aacC1* was cloned into pUCP26 (169). Then, the *syrB1*::*uidA-aacC1* constructs were isolated as *Bgl*I, *Nar*I, and *Drd*I fragments, polished, and cloned into the dephosphorylated *Sma*I site of pUCP26 to generate pNW104-1, pNW104-2, and pNW104-3, respectively (Table 3.1).

To further define the promoter region of *syrB1*, a *Hin*dIII site was introduced at a different location in the upstream region of *syrB1* using a QuikChange® Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer's directions. Primers B8SDM1F and B8SDM1R were used for construct pNW104-4, while B8SDM2F and B8SDM2R were used for construct pNW104-5. Constructs pNW104-4

and pNW104-5, with different promoter regions, were obtained by subcloning the *Hin*dIII-*Eco*RI fragments into pUCP26. Similarly, to test whether the effect of deletion of the upstream sequence from the -42 region on expression of the *syrB1*::*uidA* reporter, primers B8SDM6F and B8SDM6F were used to generate plasmid pNW104-8. By deletion of the upstream sequence from the -42 region, 13-bp of the total 20-bp of the *syr-syp* box was deleted in the promoter region of operon IV. Plasmid pNW104-4 was used as a template, in conjunction with primers B8SDM3F and B8SDM3R, to generate pNW104-6, in which the -10 region (TGAAAT) was substituted with CTGCAG. Similarly, plasmid pNW104-7 was generated with primers B8SDM4F and B8SDM4R, in which the -35 region (GACAGACGC) was changed to CTGCAG. To further test the effect of mutation of the *syr-syp* box, primers B8SDM5F and B8SDM5R was used to generate plasmid pNW104-9, in which TGTCCC of at the 5' end of the *syr-syp* box was replaced with CTGCAG. All site-directed mutations were verified by DNA sequencing using the primer syrB1PE. The plasmids used for GUS assays are listed in Table 3.1.

To identify the promoter region of operon III, 5' *cis* promoter deletion constructs were made from pSL105, a transcriptional fusion construct of the *sypA* gene with a promoterless *uidA* gene insertion in the *Bst*Z17I site. To construct pSL105, the 3.2-kb *Hin*dIII-*Bgl*I fragment containing the *uidA-aacC1* genes from pSL2 was polished with T4 DNA polymerase and inserted at the *Bst*Z17I of pBS008 to generate pSL103. Then, the *Eco*RI-*Hin*dIII fragment-containing portion of the *syrD*/*sypA*::*uidA-aacC1* fusion was subcloned into pUCP26. The resulting construct, pSL105, lacks the promoter region for expression of the *sypA* gene and was used to define the promoter by inserting

different upstream fragments. Variable regions of the operon III promoter, from the *Eco*RI site of *syrD* to the regions upstream or downstream of *syrP,* were amplified by PCR. The forward primer, syrPR6, was used for all amplifications. Reverse primers containing an *Eco*RI restriction site were paired with syrPR6 for amplification of promoter variants of operon III. The resulting amplicons were digested with *Eco*RI and cloned into the *Eco*RI site of pSL105. All constructs were verified by sequencing using primer syrDF7 to confirm that the orientations are correct. The reverse primers used to generate the constructs were syrCRTR1 (pNW105), syrBR11 (pNW105-1), syrBR24 (pNW105-2), syrPR15 (pNW105-3) (Table 3.2).

GUS assays

The effects of *cis* elements on expression of the *syrB1::uidA* or *sypA::uidA* gene fusion were evaluated by fluorometric analysis of GUS activity. The GUS assays were performed as described previously (101). All assays for GUS activity were performed three times on separate days with duplicate cultures for each treatment.

Mutagenesis of the *rpoS* gene

The *rpoS* gene was amplified by PCR with Vent DNA Polymerase (New England Biolabs, Beverly, MA). The primers used for amplification were rpoSF1 and rpoSR1 (Table 3.2). The resulting *rpoS* fragment was separated by gel electrophoresis and purified from the agarose gel using the QIAEX II Gel Extraction Kit (QIAGEN Inc., Valencia, CA) and cloned into the pGEM®-T Easy Vector (Promega Corporation, Madison, WI) to generate pGEMTrpos. The *rpoS* gene was disrupted by a nonpolar mutation with the *nptII* gene cloned into the unique *Cla*I site

on pGEMTrpos. The disrupted *rpoS* fragment was then isolated with *Not*I, and dephosphorylated. Plasmid pBR325 was linearized with *Eco*RI, dephosphorylated and polished, and the mutant *rpoS* fragment was inserted into pBR325 by blunt-end ligation to generate pBR325rposKm.

Construction of B301D *rpoS* mutant by marker-exchange mutagenesis

In order to generate an *rpoS* mutant of B301D, plasmid pBR325rpoSKm was introduced into B301D competent cells by electroporation for marker-exchange mutagenesis, as described previously (178). The resultant mutant B301DNW201 was confirmed by Southern hybridization.

Bioassays of *P. syringae* pv. *syringae* strains for syringomycin and syringopeptin production

The *P. syringae* pv. *syringae* strains were tested for the ability to produce syringomycin and syringomycin using previously described bioassays (146). The fungus *Geotrichum candidum* F-260 and the bacterium *B. megaterium* Km were used to detect the production of syringomycin and syringopeptin, respectively, on PDA agar medium. The *P. syringae* pv. *syringae* strains were cultured on plates at 25°C for 4 days and then sprayed with *G. candidum* or *B. megaterium* and cultured overnight. The bioassays were repeated on three independent occasions with duplicate cultures for each time.

RESULTS

Operon analysis of the *syr-syp* genomic island

A total of five operons were identified in the *syr-syp* genomic island (Fig. 3.1 and Table 3.3). Of the 1 16 of the synthesis and secretion genes associated with responsible

TABLE 3.3. Operons identified in the *syr-syp* genomic island

Operon	Genes	Size (kb)
Operon I	*pseA-pseB-pseC*	5.7
Operon II	ORF19-*sypD*	3.2
Operon III	*syrP-syrD-sypA-sypB*	35.6
Operon IV	*syrB1-syrB2-syrC*	4.4
Operon V	*syrF*-ORF20	2.3

for syringomycin and syringopeptin production, 14 were organized into operons including *pseA-pseB-pseC* (operon I), *sypD*-ORF19 (operon II), *syrP-syrD-sypA-sypB* (operon III), *syrB1-syrB2-syrC* (operon IV), and *syrF*-ORF20 (operon V). The size of the operons ranged from 2.3-kb for operon IV to 35.6-kb for operon III. Operon III appeared to be one of the biggest operons identified in bacteria (Fig. 3.1 and Table 3.3). *syrP*, *syrD*, *sypA*, and *sypB* are transcriptionally joined as demonstrated by RT-PCR analysis (Fig. 3.2, lanes 1, 4, 9, 13 and 14). To further confirm that there is no individual transcriptional start site for *sypA*, primer extension analyses were carried out with primers sypAextensionr17745, sypAPER1, and sypAPER2 (Table 3.2), which are complementary to regions -69 to -48, -22 to -5, and +72 to +95 in relation to the initiation codon of *sypA*, respectively. No cDNA product was observed for any of the three primers. No *sypB-sypC* product was obtained with primers sypBRTF andsypCRTR (Fig. 3.2, lane 10). To ensure that negative RT-PCR data were not the results of internal problems with primers, genomic DNA was used as a template to check the fidelity of the primers. For all of the primer pairs, PCR products were obtained as predicted (data not shown). These results demonstrate that *syrP*, *syrD*, *sypA*, and *sypB* are in one operon.

Besides the five operons, *dat*, *sypC*, *syrE*, ORF21, and ORF22 are transcribed individually based on RT-PCR analysis (Fig. 3.2, lanes 2, 5, 6, 8, 10, 17 and 18). Two typical rho-independent terminators, located after the *syrP-syrD-sypA*-s*ypB* operon and the *syrB1-syrB2-syrC* operon, were identified by the FindTerm program (http://www.softberry.com, Fig. 3.1). Two genes within the island, *syrG* and *salA,* were

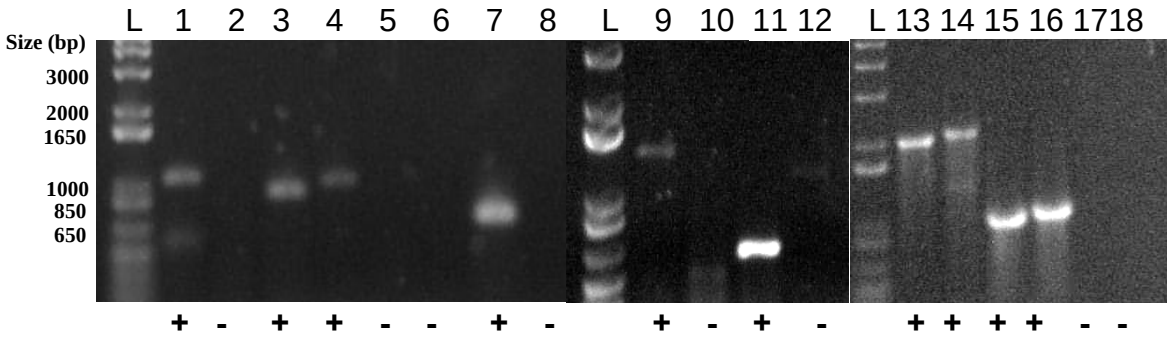


FIG. 3.2. RT-PCR analysis to define the operons in the *syr-syp* genomic island. RT-PCR analysis was performed with total RNA isolated from *Pseudomonas syringae* pv. *syringae* incubated on PDA medium for 3 days at 25^{o}C and submitted to agrose gel electrophoresis. The RT-PCR products were named after the ORFs that they cover and are numbered as follows: 1, *syrP*-*syrD*; 2, *sypC*-*sypD*; 3, ORF19-*sypD*; 4, *syrB2*-*syrC*; 5, *sypC*-ORF19; 6, *syrC*-*syrE*; 7, *syrF*-ORF20; 8, *syrE*-ORF20; 9, *sypA*-*sypB*; 10, *sypB*-*sypC*; 11, *syrB1*-*syrB2*; 12, *pseB*-ORF14; 13, *sypA*-*syrP-1*; 14, *sypA*-*syrP-2*; 15, *pseA*-*pseB*; 16, *pseB*-*pseC*; 17, *dat*-*sypD*; 18, *cmaU*-ORF22. A "+" indicates that two genes are in one operon, whereas a "-" indicates that two genes are not in one operon. The "L" represents lanes with a DNA ladder.

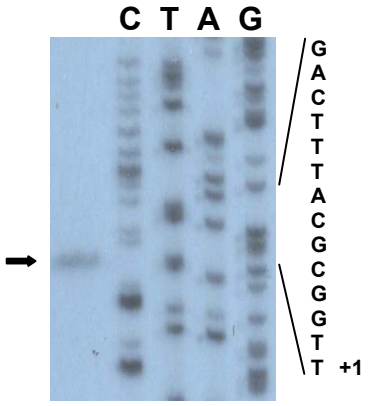


FIG. 3.3. Primer extension analysis of total RNA from *P. syringae* pv. *syringae* B301D to define the promoter region of operon IV (*syrB1-syrB2-syrC*). The 104-bp cDNA products (indicated by a black arrow) of operon IV mapped the transcriptional start site to a thymine residue shown with +1.

Operon III TTGCGGTGACAGACGCCTTAGGCCATCAGTGTTTTTATTC**T**

Operon IV CTCGACAGACGCAGCCCGTTAGTCGGTGCTGAAATGCGCCA**A**

salA GTTTGCCCACAGGACTCAAAGAATACAAAACACTTACTTA**T**

Sigma 70 TTGACA TATAAT

-35 -10

FIG. 3.4. Comparison of putative promoter sequences of the *syr-syp* genes. A: Promoter sequence of operons III, IV and *salA* gene predicated based on the defined transcriptional start sites. Conserved sequence motifs corresponding to the -35 and -10 sites are underlined. The typical sigma 70 dependent promoter sequence is listed at the bottom.

not analyzed with RT-PCR, but it is predicted that they are independent genes, bringing the total number of transcriptional units for the *syr-syp* genomic island to 12 (Fig. 3.1).

Determination of the transcriptional start sites and promoters of the *salA* gene and operons III and, IV and the *salA* gene

Once the transcriptional units of the *syr-syp* genomic island were identified, the transcription start sites of the *salA* gene and operons III and, IV, and the *salA* gene were defined by primer extension analysis. A transcript was obtained for operon IV with primer syrB1PE, which is complementary to the -20 to +3 region in relation to the start codon of operon IV, indicating a transcriptional start site 104 nucleotides upstream of the *syrB1* translational start codon (Fig. 3.3). For operon III, primer extension indicated a transcriptional start site 75 nucleotides upstream of the start codon of *syrP* (data no shown). This analysis was performed using primer syrPPE, which is complementary to -37 to -14 region upstream of the initiation codon of operon III. For the *salA* gene, the transcriptional start site was located 63 nucleotides upstream of the *salA* translational start, using primer salAPE, which is complementary to -34 to -11 region in relation to the start codon of *salA* (data not shown).

The transcript of operon III was initiated at a thymine residue 75-bp upstream of the translational start site of *syrP*. The transcriptional start site of operon III suggested a putative promoter region, GTGACAN$_{18}$TGTTTT (Fig. 3.4). Similarly, the transcriptional start site of operon IV was located to an adenine residue 104-bp upstream of *syrB1* and suggested a putative promoter region CAGACGN$_{18}$TGAAAT (Fig. 3.4). Both promoters share high similarity to the sigma 70 consensus promoter sequences of

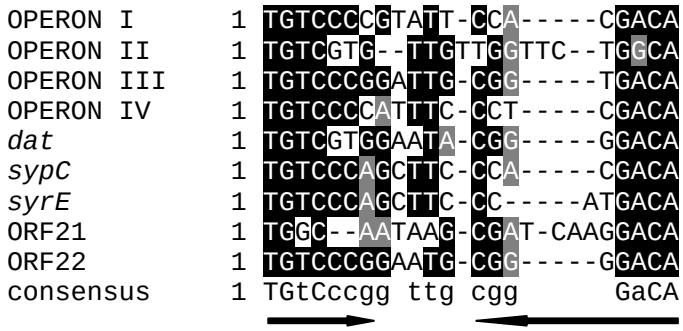


FIG. 3.5. Alignment of the *syr-syp* box elements in B301D. The dyad-symmetric DNA regions around the -35 region of the *syr-syp* genes were aligned using T-COFFEE and conserved sites are highlighted. The inverted repeats are indicated by arrows. The boxshade figure was generated with BOXSHADE 3.21 (www.ch.embnet.org/software/BOX_form.html).

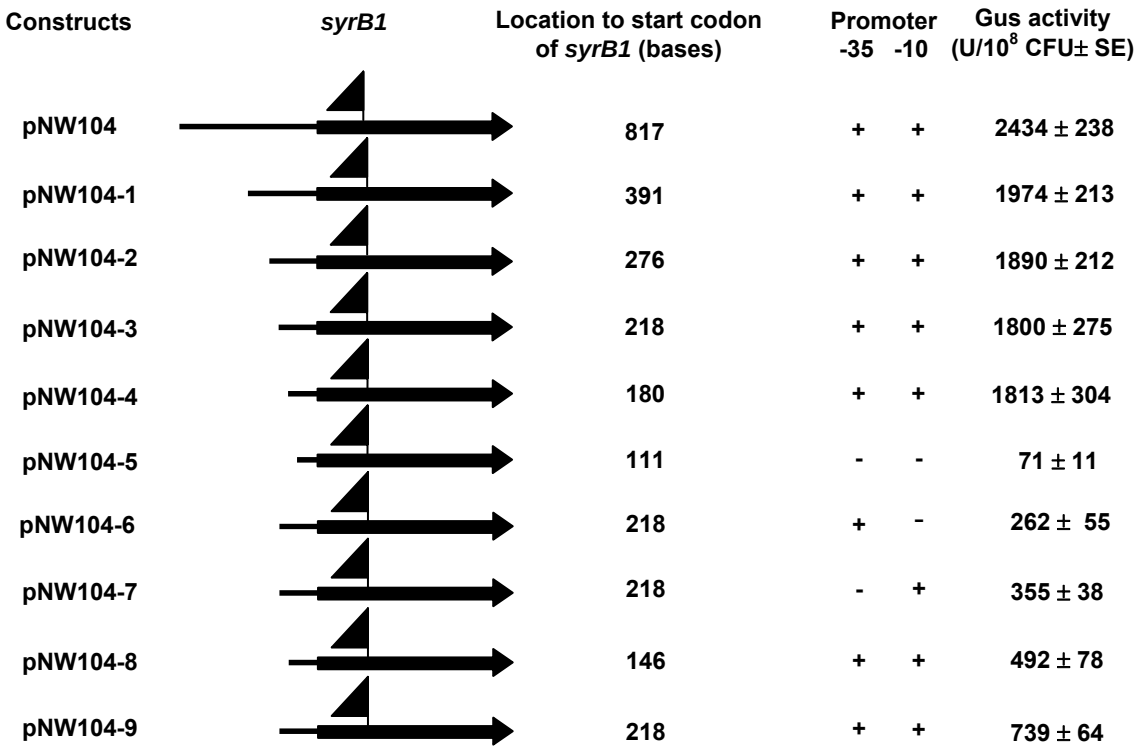


FIG. 3.6. Deletion analysis of the promoter region of operon IV by testing the effect of cis elements on expression of a *syrB1*::*uidA* transcriptional fusion. The *uidA* construct was inserted into the *Eag*I site of *sypA*. The *uidA* construct is represented by a flag. Plasmids containing the *syrB1*::*uidA* fusion with specific deletions or mutations were electroporated into cells of strain B301D to test β-Glucuronidase (GUS) activities. The transcription start site for operon IV was mapped at a position 104 nucleotides upstream of the *syrB1* start codon. All assays were repeated on three occasions with duplicate cultures.

gram-negative bacteria (22). The transcript of *salA* was initiated at a thymine residue 63-bp upstream of the translational start site of *salA*. The transcriptional start site of *salA* suggested a promoter region CCCACAN$_{17}$AACACT, which also shares some similarity with the typical sigma 70 consensus promoter sequences (22).

Identification of the *syr-syp* box

One imperfect dyad symmetric sequence (20-bp in length) was observed to overlap with the -35 regions of operon III based on computer analysis (Fig. 3.5). Similar sequences were found in the potential promoter regions of the *syr-syp* genes and operons responsible for synthesis and secretion of syringomycin and syringopeptin (Fig. 3.5). The consensus sequence TGTCccgN4cggGACA is named the "*syr-syp* box".

Deletion and site-directed mutagenesis analysis of the promoter region of operon IV

The effects of the mutation of the *cis* elements on expression of the *syrB1*::*uidA* fusion are shown in Fig. 3.6. Deletion constructs were generated from 817-bp (pNW104), 391-bp (pNW104-1), 276-bp (pNW104-2), 218-bp (pNW104-3), 180-bp (pNW104-4), and 111-bp (pNW104-5) upstream of the translational start site of *syrB1*. The resulting GUS activities of these constructs in B301D were: 2434, 1974, 1890, 1800, 1813 and 71 U/10^8 CFU, respectively. This indicates that the 69-bp region from 111- to 180-bp upstream of the start codon of *syrB1* is critical for expression of the *syrB1*::*uidA* fusion. When the putative -10 region TGAAAT was substituted with CTGCAG, expression of the *syrB1*::*uidA* reporter in B301D was decreased about 85%. When the putative -35 region GACAGACGC was replaced with CTGCAG, expression of the *syrB1*::*uidA* fusion in B301D was reduced about 80%. Once the -10 and -35

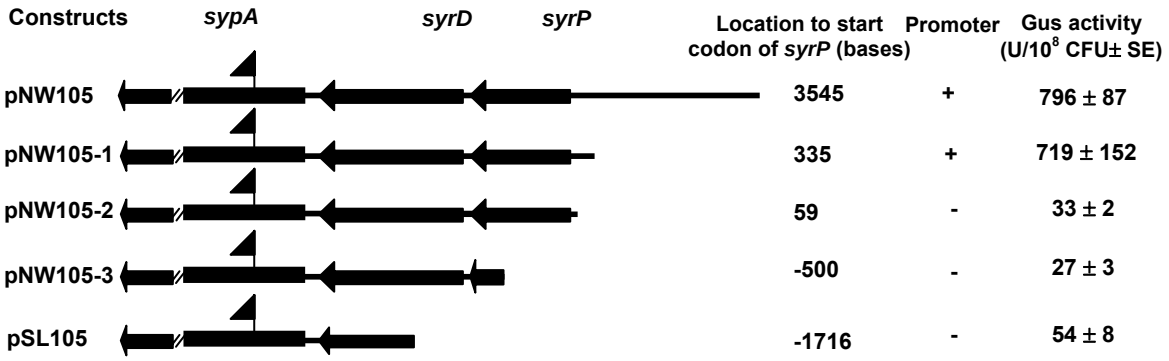


FIG. 3.7. Deletion analysis of the promoter region of operon III by evaluation of the effect of cis elements on expression of a *sypA*::*uidA* transcriptional fusion. The *uidA* construct was inserted into the *Bst*Z17I site of *sypA*. The *uidA* construct is represented by a flag. Plasmids containing the *sypA*::*uidA* fusion with specific deletions were electroporated into cells of strain B301D to test β-Glucuronidase (GUS) activities. The transcription start site for operon III was mapped at a position 75 nucleotides upstream of the *syrP* start codon. All assays were repeated on three occasions with duplicate cultures.

regions were confirmed by deletion analysis, the effect of the *syr-syp* box on expression of the *syrB1*::*uidA* fusion was tested by deletion from the -42 region and mutation of the 5' end of the *syr-syp* box. When the upstream sequence of the -42 region was deleted, expression of the *syrB1*::*uidA* fusion in B301D was reduced about 73%. Expression of the *syrB1*::*uidA* reporter was lowered 59% when TGTCCC of the *syr-syp* box was replaced with CTGCAG.

Deletion analysis of the promoter region of operon III

Effects of the deletions of *cis* elements on expression of the *sypA*::*uidA* fusion are shown in Fig. 3.7. When the upstream sequence was intact as shown in pNW105 and pNW105-1, the GUS activities were 796 and 719 U/10^8 CFU, respectively. Expression of the *sypA*::*uidA* reporter was decreased 94% when the upstream sequence was deleted from 59-bp upstream of the translational start site of operon III. Based on the deletion analysis, the promoter region was defined to a region between 59- to 335-bp upstream of the translational start site of operon III. Further deletion analysis of the downstream sequence from the translational start site of operon III showed results similar to pNW105-2. The GUS activities for pNW105-3 and pSL105 were 27 and 54 U/10^8 CFU, respectively.

Effect of mutagenesis of *rpoS* on the production of syringomycin and syringopeptin by *P. syringae* pv. *syringae* strain B301D

A nonpolar mutation was constructed in the *rpoS* gene by insertion of an *nptII* cassette downstream of the *rpoS* start codon between nucleotides 35 and 36. Southern analysis confirmed the disruption of *rpoS* in the B301D genome (data not shown). No

detectable difference was observed between syringomycin and syringopeptin production by wild type strain B301D and the *rpoS* mutant strain based on bioassays (data not shown). This shows that *rpoS* is not required for syringomycin and syringopeptin production. The bioassays were repeated three times with consistent results.

DISCUSSION

Conservation of the promoter regions in bacteria is vital for the bacteria to coordinated expression of related genes of complementary pathways in response to the environment. Expression of the *syr-syp* genes are coordinately controlled by plant signal molecules through the SalA/SyrF pathway in *P. syringae* pv. *syringae* B301D (103). It was established in this study that the conserved sequences, including the -10/-35 sequence and the *syr-syp* box, in the promoter regions of the *syr-syp* genes contribute to the co-regulation of syringomycin and syringopeptin production. The -10/-35 sequence and the *syr-syp* box were required for expression of the *syrB1*::*uidA* reporter and similar sequences have been identified in the upstream region of other *syr-syp* genes and operons, which are responsible for the biosynthesis and secretion of syringomycin and syringopeptin.

The first step to understand the regulatory mechanism of co-regulation of the *syr-syp* genes was to determine the operon structure of the *syr-syp* genomic island. The 132-kb *syr-syp* genomic island was divided into five operons and seven individual genes (Fig. 3.1 and Table 3.3). For the five operons in the *syr-syp* genomic island, the intergenic lengths range from -4- to 241-bp. This is consistent with the fact that adjacent open reading frames within an operon are usually separated by no more than 300-bp

(46). In fact, the start codon of ORF20 overlaps with the stop codon of *syrF* (Fig. 3.1). This overlap provides strong evidence that *syrF*-ORF20 belong to one operon (Fig. 3.1) (92). In addition, operons usually contain genes that are functionally related to each other (46, 109). Operon II (i.e., *sypD* and ORF19) is predicted to encode an ATP-binding cassette (ABC) transporter system with *sypD* encoding a periplasmic membrane fusion protein and ORF19 encoding a cytoplasmic membrane protein (83). Operon IV (i.e., *syrB1*, *syrB2*, and *syrC*) is involved for in the synthesis of syringomycin, with *syrB1* and *syrB2* encoding synthetase proteins, and *syrC* encoding a thioesterase protein. Previous studies demonstrate that operons are often conserved among bacterial lineages (77, 109). This is true for operon I (i.e., *pseA*, *pseB*, and *pseC*) because BLAST search results (6) indicate that the *pseA-pseB-pseC* operon is conserved among many bacterial genomes, including *Dechloromonas aromatica Ralstonia solanacearum* and *Ralstonia solanacearum Dechloromonas aromatica*(139). Rho-independent terminator is widely used to predict the end of one transcription unit (48, 93). In this study, rho-independent terminators were found downstream of the stop codons of *sypB* and, *syrC*, *salA*, and *syrG* (101) respectively (Fig. 3.1). Besides the five operons mentioned above, seven genes (i.e., *dat*, *sypC*, *syrE*, ORF21, ORF22, *syrG*, and *salA*) are transcribed as monocistronic units, as suggested by the RT-PCR experiments and computer analysis. These data demonstrate that the *syr-syp* genomic island contains at least 12 different promoter regions.

The promoters of the *syr-syp* genes and operons share high similarity with each other. Both promoters of operons III and IV share high similarity with the typical

sigma 70-like promoters (122). Sigma 70-like promoter sequences were observed for all of the *syr-syp* genes and operons (data not shown). More interestingly, a 20-bp sequence showing imperfect dyad symmetry was observed around the -35 region of *syrB1* and was required for expression of the *syrB1*::*uidA* fusion (Fig. 3.6). Expression of the *syrB1*::*uidA* fusion in B301D was reduced about 73% when the upstream sequence of -42 region was deleted in pNW104-8, in which 13-bp of the *syr-syp* box from the 5' end was removed, (Fig. 3.5 and Fig. 3.6). Expression of the *syrB1*::*uidA* reporter was lowered 59% when TGTCCC of the *syr-syp* box was replaced with CTGCAG (Fig. 3.5 and Fig. 3.6). This sequence might be the key regulatory sequence for the co-regulation of all of the *syr-syp* genes, because this conserved sequence has been found in the promoter regions of other *syr-syp* genes/operons. The conserved inverted repeat sequence TGTCccgN4cggGACA, which overlaps with the -35 region, is predicted to be the binding site of the SyrF protein. SyrF belongs to the LuxR-FixJ family and shares significant homology with the 2.4 region of RNA polymerase sigma factor 70, which interacts with the DNA at the -35 region (101, 122). Additionally, the feature of dyad symmetry of the *syr-syp* box is consistent with the fact that LuxR family proteins form dimers and bind to the inverted repeat sequence overlapping with the -35 region (29, 43). The fact that SyrF forms dimers in vitro (Wang and Gross, unpublished data) further supports the idea that the *syr-syp* box with its dyad symmetry is the binding site for SyrF. It is not surprising that the promoter regions of *salA*, *syrF*, and *syrG* do not contain the consensus sequence as operons III and IV, since *salA*, *syrF*, and *syrG* do not belong to the SyrF regulon (Wang and Gross unpublished). For the *syr-syp* promoters

analyzed, there was great disparity in the extent to which each promoter resembles the consensus sequence, except for the eight nucleotides at the end of the *syr-syp* box (Fig. 3.4). A similar sequence with imperfect dyad symmetry has been reported for the TnrA box (TGTNANAWWWTMTNACA) in *B. subtilis* (175). In addition, *las-rhl* box-like sequences (a 20-bp imperfect inverted repeat sequence) were identified in the promoter regions of 73 quorum-sensing-controlled genes in *Pseudomonas aeruginosa* using computer analysis (Martin et al. 2003).

The sigma factors involved in the regulation of the *syr-syp* genes and operons are yet to be determined. Apparently, sigma 54 is not involved in the regulation of the *syr-syp* genes/operons because the conserved "-24(GG)/-12(GC)" feature (23) was not found for the *salA* gene, operon III or IV, or the *salA* gene. Originally, it was thought that sigma 38 might be the sigma factor involved in controlling the production of syringomycin and syringopeptin since it controls the transcription of a considerable number of secondary metabolites (73). Nevertheless, the fact that disruption of *rpoS* did not affect the production of syringomycin and syringopeptin indicates that *rpoS* is not the sigma factor responsible for transcriptional initiation of for the *syr-syp* genes. Sigma 70 might be the sigma factor responsible for the transcription of the *syr-syp* genes (122). The primary sigma factor sigma 70 is involved in the production of antibiotics and 2, 4-diacetylphloroglucinol by *Pseudomonas fluorescens* CHA0 (145). In addition, LuxR interacts with sigma 70 to recruit RNA polymerase to the *luxI* promoter (Stevens et al. 1999). However, it is still possible that some alternative sigma factors belonging to the sigma 70 family recognize the *syr-syp* promoters (63).

In conclusion, the 20-bp consensus sequence with imperfect dyad symmetry is required for expression of the *syrB1* gene and was observed in the co-regulated *syr-syp* genes responsible for synthesis and secretion of syringomycin and syringopeptin. Additional studies are needed need to be done to figure out the sigma factors involved in the regulation of the *syr-syp* genes. It is crucial to determine the interaction of the *syr-syp* box with two key activators SalA and SyrF for understanding the regulatory mechanism of the *syr-syp* genes. The characterization of the activators SalA and SyrF will help in understanding the regulatory mechanism of the *syr-syp* genes. A full appreciation of the regulation of syringomycin and syringopeptin production will shed light on at least one of the mechanisms that *P. syringae* uses to coordinate expression of distinct virulence factors.

CHAPTER IV

CHARACTERIZATION OF THE TRANSCRIPTIONAL ACTIVATORS SalA AND SyrF, WHICH ARE REQUIRED FOR SYRINGOMYCIN AND SYRINGOPEPTIN PRODUCTION BY *P. syringae* pv. *syringae*

OVERVIEW

Production of the phytotoxins syringomycin and syringopeptin by *P. syringae* pv. *syringae* is controlled by the regulatory genes *salA* and *syrF*. Analysis with 70-mer oligonucleotide microarrays established that the *syr-syp* genes responsible for synthesis and secretion of syringomycin and syringopeptin belong to the SyrF regulon. Vector pMEKm12 was successfully used to express both SalA and SyrF proteins fused to a maltose-binding protein (MBP) in *E. coli* and *P. syringae* pv. *syringae*. Expression of MBP-SalA and MBP-SyrF partially restored syringomycin production by a *salA* mutant and a *syrF* mutant, respectively. Both the MBP-SalA and MBP-SyrF fusion proteins were purified by maltose-affinity chromatography. Gel shift analysis revealed that the purified MBP-SyrF, but not the MBP-SalA fusion protein, bound to a 262-bp fragment containing the *syr-syp* box. Purified MBP-SalA caused the shift of a 324-bp band containing the putative *syrF* promoter. Gel filtration analysis or cross-linking experiments indicated that both SalA and SyrF form homodimers in vitro. Syringomycin production by B301D was decreased and β-Glucuronidase activities (GUS) of the *sypA*::*uidA* and *syrB1*::*uidA* reporters was reduced 58% and 66%, respectively, by overexpression of the N-terminal region (681-bp) of SalA. The effect of

SalA on expression of the *syr-syp* genes is mediated by SyrF, which activates the *syr-syp* genes by directly binding to the promoter regions. Both SalA and SyrF function similarly to other LuxR family proteins in dimerization and interaction with promoter regions of target genes.

INTRODUCTION

Syringomycin and syringopeptin production by *P. syringae* pv. *syringae* is coordinately controlled by a common regulatory mechanism. Both toxins are lipodepsipeptides and synthesized separately by modular nonribosomal peptide synthetases (64, 147, 177). Genes dedicated to biosynthesis, secretion, and regulation of the two toxins are localized in the syringomycin (*syr*) and syringopeptin (*syp*) genes clusters, which are adjacent to one another on the chromosome (101, 147). Assembly of the two compounds is induced by plant signal molecules such as arbutin and D-fructose (115). Previous study demonstrated the two-component GacS/GacA system is critical for the regulation of both toxins (75, 88). The *gacS* gene encodes a transmembrane protein, which functions as a histidine protein kinase that undergoes phosphorylation in response to environmental stimuli (76). GacA is a response regulator protein that is phosphorylated by GacS (70, 136). The regulation of syringomycin and syringopeptin by GacS/GacA is mediated by the downstream regulator SalA. Neither syringomycin nor syringopeptin was produced by a *salA* mutant (75, 88, 103). Analysis with 70-mer oligonucleotide microarrays, along with β-Glucuronidase (GUS) assays and quantitative real-time PCR (QRT-PCR) analysis demonstrated that all of the *syr-syp* genes (Fig. 4.1)

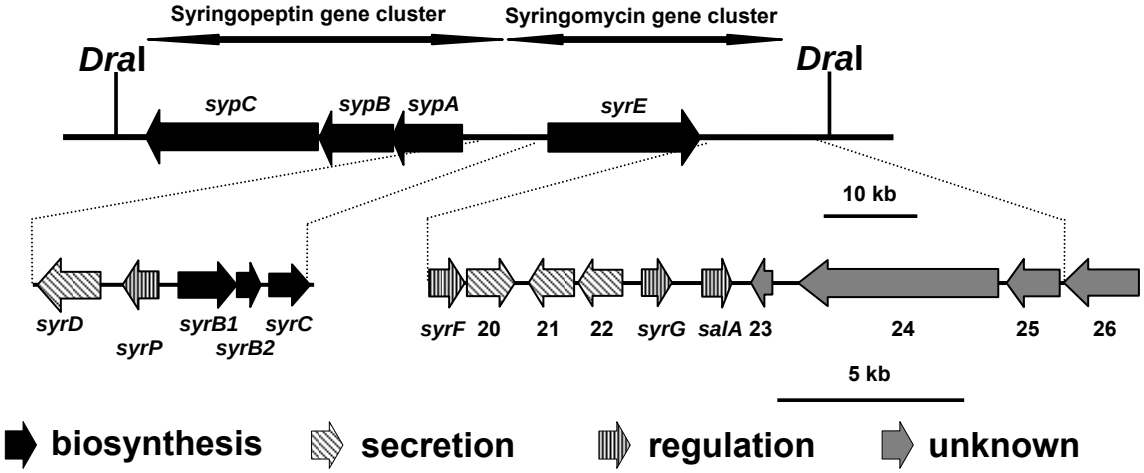


FIG. 4.1. A physical map of a 132-kb genomic island of *P. syringae* pv. *syringae* strain B301D containing both syringomycin (*syr*) and syringopeptin (*syp*) gene clusters. The positions and orientations of the known and potential open reading frames (ORFs) are shown as horizontal arrows. The solid, diagonally-striped, and vertically-striped arrows represent genes that are predicted to be involved, respectively, in the synthesis, regulation, and secretion of the phytotoxins. The gray arrows represent the potential ORFs for which functions remain unknown.

belong to the SalA regulon (103). The *syrF* gene, which is positively controlled by *salA*, is also required for syringomycin and syringopeptin production (101). Consequently, both SalA and SyrF are critical for the co-regulation of syringomycin and syringopeptin production.

Both SalA and SyrF belong to a family of transcriptional activators characterized by high sequence similarities to the LuxR helix-turn-helix (HTH) domain in the C-terminal region (84). The LuxR DNA-binding domain consists of four-helix bundles, in which the HTH motif comprises the second and third helices (53). The LuxR superfamily proteins were grouped into two major subfamilies on the basis of sequence similarity at the N-terminus and by their functional regulatory mechanism. One subfamily consists of the autoinducer-binding regulators including LuxR (84), LasR (55), CarR (168), EsaR (165), CerR (129), and TraR (127), which are activated by homoserine lactones. LuxR protein is one of the most studied autoinducer-binding regulators and is essential for quorum sensing in *Vibrio fischeri* (54). LuxR contains an autoinducer-binding domain at the N-terminus that interacts with an acyl-homoserine-lactone (acyl-HSL) and the HTH DNA binding motif at the C-terminus (50). LuxR activates the *lux* operon, necessary for light generation, by binding to the 20-bp *lux* box centered at -42.5 position relative to the *luxI* transcriptional start site (43). Accordingly, LuxR contacts both the α-subunit carboxy-terminal domain (αCTD) and the sigma subunit of RNA polymerase as an "ambidextrous activator" (43). Evidence indicated that LuxR functions by forming a multimer (29).

The other subfamily of LuxR-like proteins is composed of the response

regulators of the two-component signal transduction systems, including NarL (95), FixJ (9), NarP (39), GacA (138), and UhpB (80). NarL, which activates the nitrate reductase operon in *Escherichia coli*, is one of the best understood response regulators and is comprised of two domains, an amino-terminal receiver domain and a carboxyl-terminal effector domain (179). Unlike the proteins that respond to acyl-HSL, NarL is activated by phosphorylation signals (44). The NarL response regulator is phosphorylated at the N-terminal regulatory domain (179) and forms a dimer to recognize heptamer sequences, which are often present as pairs of inverted repeats, in the promoter regions of target genes (39, 107). Therefore, LuxR-type proteins function similarly in dimerization and interactions with promoter regions of target genes despite sequence differences at the N-terminus.

Sequence analyses of SalA (284 amino acids) and SyrF (276 amino acids) proteins demonstrated that both proteins contain the helix-turn-helix (HTH) DNA-binding domain of the LuxR protein family at the C-terminus (101). The C termini of SalA and SyrF exhibit 27-46% identity to LuxR (84), TraR (127), NarL (95), FixJ (9), and GerE (37, 101). Unlike LuxR, no autoinducer domain was identified at the N-termini of the SalA and SyrF proteins (54), and unlike typical response regulators, SalA and SyrF lack the "acid pocket" composed of four highly conserved residues (Asp, Asp, Asp, and Lys) characteristic of response regulator receiver domains (124). Therefore, both SalA and SyrF belong to one unique LuxR subfamily (101).

Despite evidence that *salA* and *syrF* are required for syringomycin and syringopeptin production, the mechanism behind SalA- and SyrF-activated expression of

TABLE 4.1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source
Escherichia coli		
DH10B	F⁻ *mcrA* Δ*lacX74* (ϕ80d*lacZ*ΔM15) Δ(mrr-*hsdRMS-mcrB*) *deoR recA1 endA1 araD139* Δ(*ara, leu*)7697 *galU galK* λ⁻ *rpsL nupG*	(57)
Pseudomonas syringae	pv. *syringae*	
B301D	Wild type, from pear	(61)
B301DG12	*gacS*::*nptII* derivative of B301D; Km^r	(83)
B301DSL1	*syrF*::*nptII* derivative of B301D; Km^r	(101)
B301DSL7	*salA*::*nptII* derivative of B301D; Km^r	(101)
B301DSL8	*syrB1*::*uidA-aaaC1*, derivative of B301D; Gm^r	(101)
B301DSL29	*sypA*::*uidA-aaaC1*, derivative of B301D; Gm^r	(103)
B301DNW301	Derivative of B301D with deletion mutation of *salA*	This study
Plasmid		
pGEM-T	Cloning vector; Ap^r	Strategene. La Jolla, CA
pUCP26	Cloning vector; Tc^r Ap^r	(169)
pBR325	Cloning vector; Cm^r Tc^r Ap^r	(128)
pLAFR3	Cloning vector; Tc^r	(153)
pRK415	Broad-host-range cloning vector; Tc^r	(56)
p29	pLAFR3 carrying a 16-kb fragment of *P. syringae* pv. *syringae* B301D genomic DNA; Tc^r	(101)
pSL2	pBI101 with the 0.85-kb *aacC1* gene of pUCCGM inserted at the *Eco*RI site downstream of the *uidA* gene; Km^r Gm^r	(101)
pSL8	pBR325 carrying the 3.0-kb *Eco*RI fragment of p29 containing *salA*, Tc^r Ap^r	(101)
pSL9	pBuescriptSK(+) carrying a 2.5-kb *kb Hin*dIII-*Eco*RV fragment of pSL5 containing *syrG*; Ap^r	(101)
pSL21	pBR325 carrying the 3.0-kb fragment of p29 with *nptII* insertion at the *Kpn*I site of *salA*; Ap^r Tc^r Km^r	(101)
pSL82	pMEKm12 carrying the *syrF* gene in-frame fused to the *malE*; Km^r Pip^r	Lu and Gross unpublished data
pSL83	pMEKm12 carrying the *salA* gene in-frame fused to the *malE*; Km^r Pip^r	Lu and Gross unpublished data
pNWB1probe	pGEM-T Easy Vector carrying a 262-bp DNA fragment containing the putative promoter regions of *syrB1* and *syrP*, Ap^r	This study
pNWFprobe	pGEM-T Easy Vector carrying a 324-bp DNA fragment containing the putative *syrF* promoter region, Ap^r	This study
pNWsalAkpnI	pBR325 carrying the 3.0-kb *Eco*RI fragment of p29 containing *salA* with *Kpn*I site inserted at 39-bp downstream of the start codon, Tc^r Ap^r	This study
pNWsalAkpnIKm	pBR325 carrying the *Eco*RI fragment of p29 containing *salA* with *nptII* cassette replacing the DNA fragment between the two *Kpn*I sites downstream of the start codon, Tc^r Ap^r	This study
pNWSalANE	pUCP26 carrying one *Eco*RI fragment of 1.954-kb fragment containg 1.275-kb upstream and 0.681-kb downstream of the start codon of *salA* from pSL21, Tc^r	This study

the *syr-syp* genes remains largely unknown. It is to be determined if the *syr-syp* genes are members of the SyrF regulon. In this study, it was hypothesized that both SalA and SyrF resemble LuxR proteins in dimerization and activation of the transcription of target genes by binding to the promoter regions. In particular, the control of SalA on expression of the *syr-syp* genes is mediated by SyrF, which directly binds to the promoter regions of the *syr-syp* genes and activate their expression. This study provides important foundation of understanding a unique LuxR subfamily of proteins.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media

P. syringae pv. s*yringae* strains and plasmids used in this study are listed in Table 4.1. Strains were routinely cultured in nutrient broth-yeast extract (NBY) broth or on NBY agar medium (164) at 25°C (*P. syringae* pv. s*yringae*) or in Luria broth (LB) or on LB agar medium at 37°C (*E. coli* strain DH10B) (57). For microarray analysis, *P. syringae* pv. s*yringae* strains were cultured on syringomycin minimal medium with exogenously added arbutin (100 μM) and D-fructose (0.1%) (SRM_{AF}) (62). For GUS assay experiments, *P. syringae* pv. s*yringae* strains were cultured in potato-dextrose broth (PDB) medium. Antibiotics (Sigma Chemical Co., St. Louis, MO) were added to media in the following concentrations: 25 μg of tetracycline per ml, 100 μg of kanamycin per ml, 100 μg of ampicillin per ml and 5 μg of gentamycin per ml.

Microarray analysis

To test the effect of mutation of *syrF* on the transcriptional expression of the *syr-syp* genes and representative genes associated with plant pathogenesis of *P. syringae*

pv. *syringae*, microarray analysis was performed as described previously (103). Wild type strain B301D and *syrF* mutant B301DSL1 of *P. syringae* pv. *syringae* were cultured with shaking at 25°C overnight in SRM_{AF} liquid medium (2 ml). Cells were harvested by centrifugation, washed twice, and then diluted with sterile distilled water to a concentration of approximately 2 x 10^8 CFU per ml. Cell suspensions (50 µl) were spread on SRM_{AF} plates and were incubated at 25°C for 72 h prior to recovery of cells. Total RNA was purified using a RiboPure-Bacteria Kit (Ambion, Inc., Austin, TX) following the manufacturer's instructions. Labeling of total RNA, hybridization, washing, and scanning of the microarrays were performed as described previously (103). Signal intensity and ratios were generated using GenePix Pro software and the raw data was normalized using 16S rRNA as a standard. Microarray data with intensities reproducibly higher than that of the background level were selected for analysis. Hybridization experiments were conducted two times and each slide contained duplicate arrays. Unsupervised hierarchical clustering analysis of the transcriptional profile of the *syr-syp* gene clusters and representative genes associated with virulence were performed with the self-organizing tree algorithm (SOTA) (http://www.almabioinfo.com/sota/) (74). Cluster analysis was visualized with Treeview software (www.almabioinfo.com/sota-cgi-bin/ApplyTreeView.cgi).

QRT-PCR analysis

The effect of mutation of *syrF* on expression of *sypA*, *sypB*, *syrB1*, *syrC*, *sypD*, *syrD*, *sylD*, *hrpR*, *hrpZ*, and *recA* observed in microarray analysis was verified by

TABLE 4.2. Primers used for quantitative real-time PCR analysis and PCR amplification

Name	Sequence	Resource
sypAF	TGCGGGTCGAGGCGTTTTTG	(83)
sypAR	GTTGCCGCGTCCTTGTCTGA	(83)
sypBF	TTCGATCAGGGTCACCGCCAACAATG	This study
sypBR	AGCTGCTCAATGTCGAAAAGGTC	This study
syrB1F	TTAGCGCCGCGTCAGCCCCTCTCAAG	(83)
syrB1R	GCTCAACGTCCGGGCTGCATCGCTCA	(83)
syrCF	ACCTGCAAGCGATGTTCCTC	(103)
syrCR	TGCCAGCTCGGTCTTGTTCA	(103)
sypDF	TCACCGCGATCAACGACAG CAACA	(83)
sypDR	GCAAAAGCGGCACGGGACCAAAGA	(83)
syrDF	GGAACTGCTGCCGGACCTCAA	(83)
syrDR	GC CCTCAACCGCGCACTTCAC	(83)
sylDF	ACTATCGCGCTCGTGTCCAA	(103)
sylDR	CAGCCCGATACCGTCAGAAA	(103)
recAF	CTTCGGTACGCCTGGACA	(103)
recAR	AACTCGGCCTGACGGAAC	(103)
16SF	ACACCGCCCGT CACACCA	(103)
16SR	GTTCCCCTACGGCTACCTT	(103)
syrb1RP	AGGCCTGCAGTGGACCTCAGCCCTTCACATC	This study
syrPFP	ATCCCTGCGGCCTGACGAAT	This study
syrERTF	CCCATATCGCCATCGGTAACTA	This study
syrFRP	GGGCGCTGAACAAGGAG	This study
FF-*Eco*RI	TCAGGAGTGAATTCATGAACCGACAAGTGA	Lu and Gross unpublished data
FR-*Hin*dIII	GCACAGCCAAGCTTCAATTGATCTGTTCAT	Lu and Gross unpublished data
AF-*Eco*RI	CGATGAACGAATTCCAGCTTTTCCCGCATC	Lu and Gross unpublished data
AR-*Hin*dIII	ATGCGCACAAGCTTGAACAGGGTGGTCGTT	Lu and Gross unpublished data
salA*Kpn*Isdmf	GCATCTCGGCAAAGTGGGTACCGGTATTGGAAGCCGTC	This study
salA*Kpn*Isdmr	GACGGCTTCCAATACCGGTACCCACTTTGCCGAGATGC	This study

QRT-PCR using the QuantiTect SYBR Green RT-PCR Kit (Qiagen Inc., Valencia, CA). Total RNA of wild type strain B301D and *syrF* mutant B301DSL1 were purified as described above. Primers used for QRT-PCR were designed using the Lasergene Expert Sequence Analysis Package (DNAstar, Madison, WI) and listed in Table 4.2. Primers specific for the 16S rRNA gene were used for normalization in accord with microarray analysis. QRT-PCR was performed three times as described previously (103).

Construction of plasmids pSL82 and pSL83 for expression of the MBP-SyrF and MBP-SalA fusion proteins

To express the SyrF and SalA proteins, genes *syrF* and *salA* were amplified with PCR and cloned into vector pMEKm12 (101) to generate pSL82 and pSL83 (Table 4.1), respectively. The primer pairs used for amplification of *syrF* and *salA* were FF-*Eco*RI and FR-*Hin*dIII, and AF-*Eco*RI and AR-*Hin*dIII, respectively (Table 4.2). The amplified fragments contained *Eco*RI and *Hin*dIII restriction sites and were digested with *Eco*RI and *Hin*dIII for insertion into pMEKm12 to generate pSL82 and pSL83 for overexpression of SyrF and SalA, respectively.

Expression and purification of SyrF and SalA proteins

Proteins were expressed in *E. coli* strain DH10B by addition of isopropyl-β-D-thiogalactopyranosid (IPTG, 0.3 mM) and purified with maltose affinity chromatography according to the manufacturer's instructions (New England Biolabs, Beverly, MA). Protein concentrations were measured using the Bradford assay (20). Fusion proteins were induced and purified from B301D with the same methods as described for *E. coli* except that B301D cells were induced with 5 mM IPTG

at 25°C for 6 h.

5' deletion mutagenesis of the *salA* gene

To delete the 5' end of the *salA* gene, a *Kpn*I site was introduced 39-bp downstream of the start codon of *salA* using the QuikChange® Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer's directions. The pNWsalAkpnI construct was generated with primers salAkpnIsdmf and salAkpnIsdmr (Table 4.2) and with plasmid pSL8 as the template. The *nptII* cassette was inserted into the *Kpn*I site of the resulting construct to generate construct pNWsalAkpnIKm. All the constructs were verified by DNA sequencing with appropriate primers (Table 4.2).

Construction of a B301D *salA* deletion mutant by marker-exchange mutagenesis

In order to generate a *salA* deletion mutant of B301D, the plasmid pNWsalAkpnIKm was introduced into competent cells of B301D by electroporation for marker-exchange mutagenesis as described previously (178). The resultant mutant of *salA* was confirmed by Southern hybridization.

Complementation analysis of *P. syringae* pv. *syringae* strains with bioassays

To test whether the MBP-SalA and MBP-SyrF fusion proteins are functional, constructs pSL82 (MBP-SyrF) and pSL83 (MBP-SalA) were transformed into B301DSL1 and B301DSL7, respectively. The *P. syringae* pv. *syringae* strains either with or without the expression constructs were tested for ability to produce syringomycin with standard bioassays as described previously (62). The fungus *Geotrichum candidum* F-260 was used to detect syringomycin production on

potato-dextrose agar (PDA) since syringomycin inhibits its growth. The bioassays were performed on three occasions with duplicate cultures.

Gel mobility shift assays

A 262-bp DNA fragment containing the putative promoter regions of *syrB1* and *syrP* was amplified by PCR with primers syrB1RP and syrPFP using B301D genomic DNA as template and then isolated from agarose gels. To verify the probe, the fragment was cloned into pGEM®-T Easy Vector (Promega, Madison, Wisconsin) to generate plasmid pPB1probe for sequencing confirmation with T7 primer. This fragment was end-labeled with gamma-^{32}P ATP (Perkin-Elmer Life Sciences, Inc. Boston, MA) using T4 polynucleotide kinase (Promega, Madison, WI) at 37°C for 60 min. The labeled probe (about 5 nM) was incubated with increasing amounts of MBP-SyrF or MBP-SalA for 10 min at room temperature in 10 µl of TGED binding buffer (50 mM Tris-HCl [pH 8.0], 5% [vol/vol] glycerol, 0.1 mM EDTA, 1 mM dithiothreitol) containing 20 µg of poly(dI-dC)/ml and 200 µg of bovine serum albumin/min (137). Reaction mixtures were then resolved on a 6% nondenaturing (w/v) polyacrylamide gel in Tris-borate-EDTA buffer (TBE) at room temperature at 200 V. Competition experiments were performed using 500 times more unlabeled probe as described previously (137).

Similarly, a 324-bp DNA fragment containing the intergenic region of *syrE* and *syrF* was synthesized by PCR with primers syrERTF and syrFRP, labeled with gamma-^{32}P ATP, and used to study the interaction with the purified SalA protein.

Sephacryl S-200 gel filtration

Purified MBP-SalA (1 ml) was loaded and fractionated on a column (2.5 cm in

diameter x 80 cm in length) packed with Sephacryl S-200 High Resolution (Amersham Biosciences, Piscataway, NJ) at a flow rate of 1.3 ml/min. The column was pre-equilibrated with elution buffer (50 mM Tris-HCl, pH 7.4) and calibrated with gel filtration molecular weight standards (12 to 200 kDa) (Sigma, St. Louis, MO). Eluted fractions (3 ml) were analyzed for the presence of MBP-SalA using Western-blotting. The protein content present in fractions was estimated by the Bio-Rad Protein Assay, a modification of the Bradford procedure (Bradford, 1976).

In vitro cross-linking

In vitro cross-linking experiments were performed with 20 ng/µl of purified MBP-SyrF or MBP-SalA protein in a 20 µl volume of cross-linking buffer (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4) (151). Purified MBP from *E. coli* was used as a negative control. The proteins were cross-linked for 20 min at room temperature, and the reactions were stopped by adding SDS-polyacrylamide gel electrophoresis (PAGE) buffer (0.045 M Tris-Cl, pH 6.8; 10% glycerol; 1% SDS; 0.01% bromophenol blue; 0.05 M DTT) (142) and followed by incubation for 10 min. The samples were either heated for 30 min at 37°C to maintain the formaldehyde cross-links or heated for 20 min at 95°C to destroy them before loading on a 10% SDS-PAGE gel. The samples were transferred to Hybond-P PVDF membrane (Amersham Biosciences, Piscataway, NJ) after electrophoresis, and immuno-blotted using polyclonal antibody to MBP and MBP-SyrF, respectively. Polyclonal antisera with antibodies recognizing MBP-SyrF was commercially produced by immunization of rabbits with purified SyrF protein (Pacific Immunology Corp, CA). Polyclonal antisera with antibodies for MBP

TABLE 4.3. Microarray analysis of the SyrF regulon

Genes	Ratio (S.E.M)	Gene product	References
syrD	8.22 (±1.07)	ATP-binding secretion protein	(132)
syrP	17.76 (±1.94)	Homologue of histidine kinase	(178)
syrB1	23.06 (±2.37)	Syringomycin biosynthesis enzyme	(177)
syrB2	23.04 (±2.92)	Syringomycin biosynthesis enzyme	(177)
syrC	8.56 (±1.76)	Syringomycin biosynthesis enzyme	(177)
syrE-1	2.79 (±0.46)	Syringomycin synthetase	(64)
syrE-2	2.76 (±0.47)	Syringomycin synthetase	(64)
syrE-3	2.51 (±0.28)	Syringomycin synthetase	(64)
syrF	1.31 (±0.16)	LuxR family bacterial regulator	(101)
ORF20	2.23 (±0.10)	Putative out membrane protein	(101)
ORF21	2.45 (±0.20)	Hypothetical protein	(101)
ORF22	3.27 (±0.37)	Membrane protein	(101)
syrG	1.75 (±0. 46)	LuxR family regulatory protein	(101)
salA	1.89 (±0.14)	LuxR family bacterial regulator	(101)
ORF23	0.98 (±0.14)	Hypothetical protein	(101)
ORF24-1	1.16 (±0.23)	Hypothetical protein	(101)
ORF24-2	0.83 (±0.15)	Hypothetical protein	(101)
ORF25	0.80 (±0.03)	Hypothetical protein	(101)
ORF26	0.97 (±0.14)	unknown (chemotaxic protein)	(101)
sypA	7.56 (±2.61)	Syringopeptin synthetase	(147)
sypB-1	8.49 (±0.46)	Syringopeptin synthetase	(147)
sypB-2	2.78 (±0.80)	Syringopeptin synthetase	(147)
sypC1	2.07 (±0.22)	Syringopeptin synthetase	(147)
sypC2	2.58 (±0.22)	Syringopeptin synthetase	(147)
ORF19	2.21 (±0.09)	Putative membrane protein	Kang and Gross, unpublished
sypD	2.39 (±0.32)	Putative ABC transporter	Kang and Gross, unpublished
dat	2.69 (±0.45)	Aminotransferase	Kang and Gross, unpublished
pseA	2.20 (±0.58)	Putative outer membrane protein	Kang and Gross, unpublished
pseB	1.68 (±0.33)	Efflux membrane fusion protein	Kang and Gross, unpublished
pseC	1.42 (±0.12)	Efflux membrane fusion protein	Kang and Gross, unpublished
ORF14	1.73 (±0.37)	Putative chemtaxic protein	Wang and Gross, unpublished
ORF13	1.28 (±0.27)	unknown	Wang and Gross, unpublished
ORF12	1.31 (±0.23)	Sensor protein	Wang and Gross, unpublished
ORF11	1.53 (±0.19)	Amino acid deaminoease	Wang and Gross, unpublished
ORF10	1.42 (±0.26)	Hypothetical protein	Wang and Gross, unpublished
ORF9	0.89 (±0.12)	Hypothetical protein	Wang and Gross, unpublished
ORF8	0.87 (±0.08)	Hypothetical protein	Wang and Gross, unpublished
ORF7	1.87 (±0.91)	Hypothetical protein	Wang and Gross, unpublished
ORF5	1.90 (±0.93)	Hypothetical protein	Wang and Gross, unpublished
ORF4	1.78 (±0.53)	Hypothetical protein	Wang and Gross, unpublished
ORF3	1.08 (±0.22)	Hypothetical protein	Wang and Gross, unpublished
ORF1	0.99 (±0.19)	Hypothetical protein	Wang and Gross, unpublished
hrpL	1.47 (±0.53)	alternative sigma factor	(4)
hrpR	1.38 (±0.10)	Regulatory factor	(172)
hrpS	0.94 (±0.11)	Positive regulatory factor	(172)
hrpK	1.19 (±0.13)	Unknown	Wang and Gross, unpublished
hrpJ	0.84 (±0.22)	flagellar biogenesis	(98)
hrpZ	1.76 (±0.27)	harpin	(68)

TABLE 4.3. Continued

Genes	Ratio (S.E.M)	Gene product	References
hrpW	1.36 (±0.12)	Type III effector	(26)
queA	1.36 (±0.19)	exchangeable effector locus	(4)
EEl-chp	1.14 (±0.17)	Type III chaperone protein	Wang and Gross, unpublished
hrmA	1.19 (±0.13)	Avr (effector) proteins	(4)
gacS	1.96 (±0.92)	sensor protein	(171)
gacA	1.47 (±0. 44)	regulator protein	(136)
rpoS	1.25 (±0.19)	RNA polymerase sigma factor	(113)
rpoD	1.53 (±0.12)	Principle sigma factor	(159)
rpoN	1.39 (±0.87)	putative sigma-54 protein	(2)
sigX	1.63 (±0.22)	sigma factor	(21)
psyr020094	1. 46 (±0.18)	Putative NRPS	www.jgi.doe.gov/JGI_microbial
psyr020651	0.89 (±0.13)	Putative NRPS	www.jgi.doe.gov/JGI_microbial
sylD	2.44 (±0.39)	Putative syringolin synthetase	(7)
algT	1.11 (±0.14)	Alternative sigma factor	(85)
algD	1.16 (±0.19)	GDP-mannose dehydrogenase	(47)
iaaM	1.40 (±0.22)	tryptophan monooxygenase	(108)
iaaH	1.35 (±0.34)	indoleacetamide hydrolase	(108)
ahlI	1.53 (±0.16)	acyl homoserine lactone synthetase	(41)
inaK	1.05 (±0.13)	Ice nucleation protein	(81)
pvdS	1.24 (±0.18)	Putative acetylase	(117)
pvdE	1.18 (±0.15)	pyoverdine synthetase	(110)
fur	0.99 (±0.26)	Ferric uptake regulator	(66)
lccC	1.33 (±0.08)	levansucrase	(94)
cbrB	1.48 (±0.24)	ferrisiderophore permease	(105)
cbrD	1.18 (±0.18)	ATP-binding unit in ABC transport	(105)
acsD	1.02 (±0.18)	Achromobactin biosynthetase	(52)
fsc	1.09 (±0.28)	ferric siderophore receptor	(120)
SR-dat	0.80 (±0.05)	Diaminobutyrate transaminase	(79)
tex	1.01 (±0.10)	S1 RNA binding domain protein	(120)
gshA	0.89 (±0.08)	Glutamate-cysteine ligase	(120)
argA	1.47 (±0.23)	N-acetylglutamate synthetase	(102)
argE	1.08 (±0.09)	Acetylornithine deacetylase	(102)
SA-ORF6	0.90 (±0.12)	unknown	(102)
rulA	1.19 (±0.23)	Radiation tolerance	(157)
sodB	1.78 (±0.18)	Iron-superoxide dismutase	(67)
gyrB	1.17 (±0.12)	DNA gyrase subunit B	(143)
recA	1.93 (±0.28)	Principal sigma factor	(90)
luc	1.01 (±0.13)	Luciferase	(35)
16S rDNA	1 (±0.0)	16S ribosomal RNA	(120)

* The *psyr* numbers stand for ORFs predicted by www.jgi.doe.gov/JGI_microbial.

was purchased from New England Biolabs (Beverly, MA). Western blots were performed according to the manufacturer's instructions (Amersham Biosciences, Piscataway, NJ).

Overexpression of the N-terminal region of SalA in B301D

To test the effect of overexpression of the N-terminal region of SalA, a 1.954-kb *Eco*RI fragment from pSL21 was cloned into the *Eco*RI site of pUCP26 in a forward orientation. The fragment contains 1.275-kb upstream sequence and 0.681-kb sequence downstream of the start codon of *salA*. The resultant construct, pNWSalANE, was transformed into B301D to test its effect on syringomycin production in standard bioassays. Construct pNWSalANE was transformed into B301DSL8 and B301DSL29 to test the effect of overexpression of the N-terminal region of SalA on GUS activities of the *syrB1*::*uidA* and *sypA*::*uidA* reporters. GUS assays were performed as described previously (101).

RESULTS

Identification of the SyrF regulon

Analysis of a 70-mer oligonucleotide microarray revealed that 16 *syr-syp* genes responsible for biosynthesis and secretion of syringomycin and syringopeptin were down-regulated greater than two-fold in strain B301DSL1, a *syrF* mutant, as compared with the wild type strain B301D (Table 4.3). Changes in expression levels of the synthetase genes for syringomycin (i.e., *syrB1*, *syrB2*, *syrC*, *syrE*) (Zhang et al. 1995) and syringopeptin (i.e., *sypA*, *sypB* and *sypC*) (Scholz-Schroeder et al. 2003) ranged from 2.1- to 23.1-fold. Seven putative secretion genes (i.e., *syrD*, ORF19, ORF20,

ORF21, ORF22, *sypD*, *pseA*) were repressed as much as 8.2-fold in B301DSL1. However, the fold changes of expression level for the three major regulatory genes (i.e., *salA*, *syrF*, and *syrG*), located at the right border of the *syr-syp* genomic island, were below the two-fold threshold. In addition to the genes in the *syr-syp* genomic island, the expression of *sylD*, which is responsible for biosynthesis of syringolin (Amrein et al. 2004), changed 2.44-fold.

In this study, none of the other genes or ORFs included in the array displayed more than two-fold changes in expression level (Table 4.3). Housekeeping genes, such as *sigX* (21), *algT* (85), *algD* (47), *sodB* (67), and *inaK* (96) located outside of the *syr-syp* genomic island, were expressed at high levels on SRM_{AF} media with no significant differences in expression levels between B301D and B301DSL1. Genes involved in siderophore production [i.e., *pvdS* (117), *pvdE* (110), *fsc* (120), *acsD* (52), *cbrB* (105), *cbrD* (105), and *fur* (66)], environmental stress (*rulA*) (180), quorum sensing (*ahlI*) (87), global regulation [i.e., *gacS*, *gacA*(36), *rpoN* (1), *rpoS* (72), and *rpoD* (159)], phytohormone synthesis (*iaaM*, *iaaH*) (108), and alginate production (*algD*) (91) were not affected by mutation of *syrF*.

The regulation patterns of *syrF* defined by QRT-PCR were similar to those determined by microarray analysis. QRT-PCR analyses indicated that transcriptional expression levels for *sypA*, *sypB*, *syrB1*, *syrC*, *sypD*, *syrD*, *sylD*, and *recA* changed 5.5-, 11.3-, 9.7-, 3.4-, 2.8-, 2.9-, 2.1-, and 1.2-fold, respectively, for B301DSL1 as compared to B301D grown on SRM_{AF}. Microarray analysis revealed that the transcriptional level changes for these genes were 7.6-, 5.6-, 23.1-, 8.6-, 2.4-, 8.2-, 2.4-, and 1.5-fold,

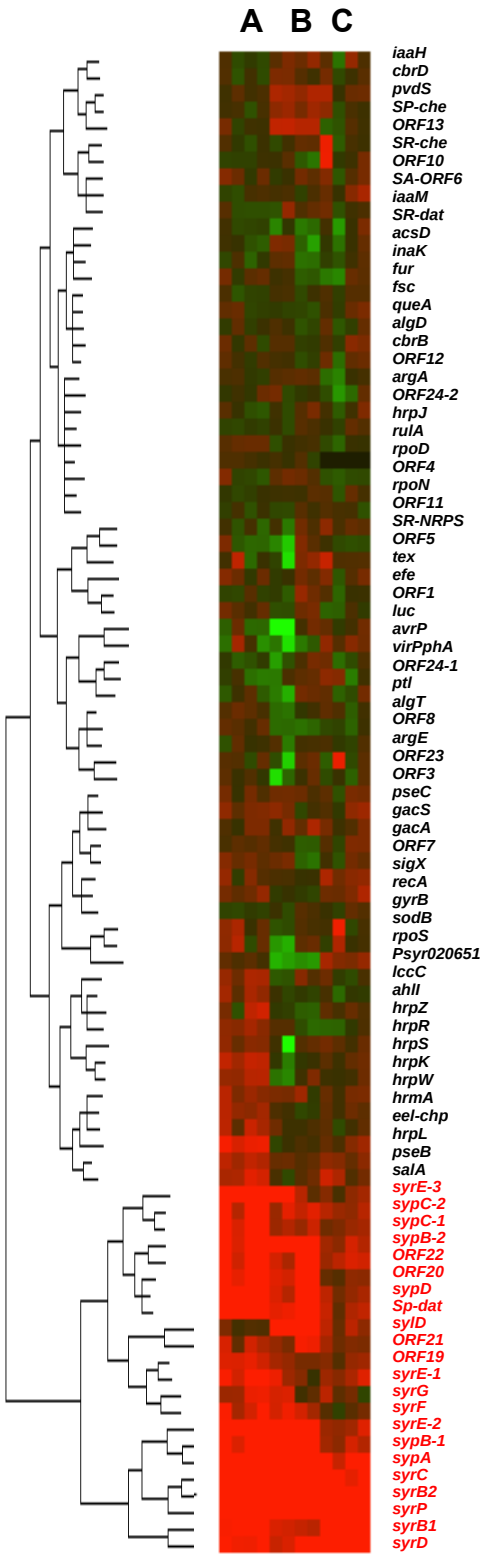


FIG. 4.2. Unsupervised hierarchical clustering analysis of the *syr-syp* and representative genes summarized in Table 4.2. Column A represents comparisons between B301D on SRM and SRM_{AF} agar media. Column B represents comparisons of gene expression between B301D and B301DSL7 (*salA* mutant). Column C compares of gene expression between B301D and B301DSL1, (*syrF* mutant). The scale of gene activities is represented from green (not induced or induced at low level) to red (induced at high level). The cluster analysis of microarray data was performed with the self-organizing tree algorithm. The *syr-syp* genes that clustered together are in red.

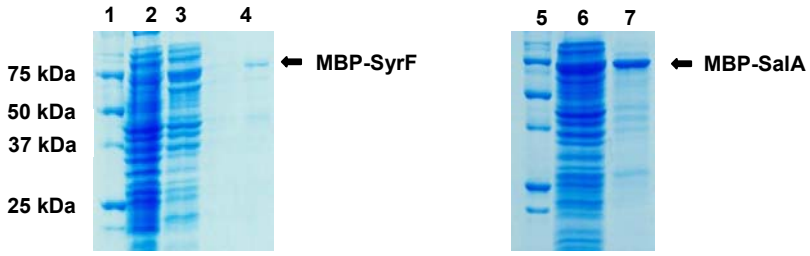


FIG. 4.3. SDS-PAGE analysis of proteins MBP-SyrF and MBP-SalA expressed in *E. coli* and purified with maltose affinity chromatography. Proteins were resolved on 10% SDS-PAGE gels at 120V and stained by Coomassie blue. Lane 1, protein standards (Bio-Rad); lane 2, DH10B, empty cells; lane 3, DH10B (MBP-SyrF); lane 4, purified MBP-SyrF; lane 5, protein standards; lane 6, DH10B (MBP-SalA); lane 7, purified MBP-SalA.

respectively (Table 4.3). QRT-PCR was repeated three times with consistent results.

The availability of transcriptional profiles for (i) B301D compared to B301DSL7 grown on PDA, (ii) B301D grown on SRM agar medium compared to that grown on SRM_{AF} agar medium, and (iii) B301D compared to B301DSL1 on SRM_{AF} agar medium facilitated cluster analysis with the self organizing tree algorithm (SOTA) (Herrero et al. 2001). As shown in Fig. 4.2, the *syr-syp* genes responsible for biosynthesis and secretion were clustered together and overlapped with the SyrF regulon.

Overexpression and purification of MBP-SyrF and MBP-SalA fusion proteins

MBP-SyrF and MBP-SalA fusion proteins were overproduced in *E. coli* strain DH10B. MBP-tagged proteins were purified by maltose affinity chromatography, and analysis of the purified proteins on a 10% SDS-PAGE gel revealed the overexpression of products approximately 75-kDa in size (Fig. 4.3). The 75-kDa size of the overexpressed proteins was expected for proteins resulting from a fusion between MBP (42.7 kDa) and SyrF (30.9 kDa) or SalA (31.2 kDa). Overexpression of MBP-SyrF or MBP-SalA in B301D generated fusion proteins similar in size to those from *E. coli* (data not shown), but the yields of overexpressed fusion proteins were much lower than in *E. coli*.

Both MBP-SyrF and MBP-SalA fusion proteins are functional in B301D

Syringomycin production was abolished for strain B301DNW301, a *salA* mutant, and reduced about 80% for B301DSL1, a *syrF* mutant, compared to that for B301D as demonstrated by the size of syringomycin zones of inhibition to *G. candidum* in bioassays (Fig. 4.4). Plasmids pSL82 (MBP-SalA) and pSL83 (MBP-SyrF) partially restored syringomycin production of strains B301DNW301 and B301DSL1 about 15

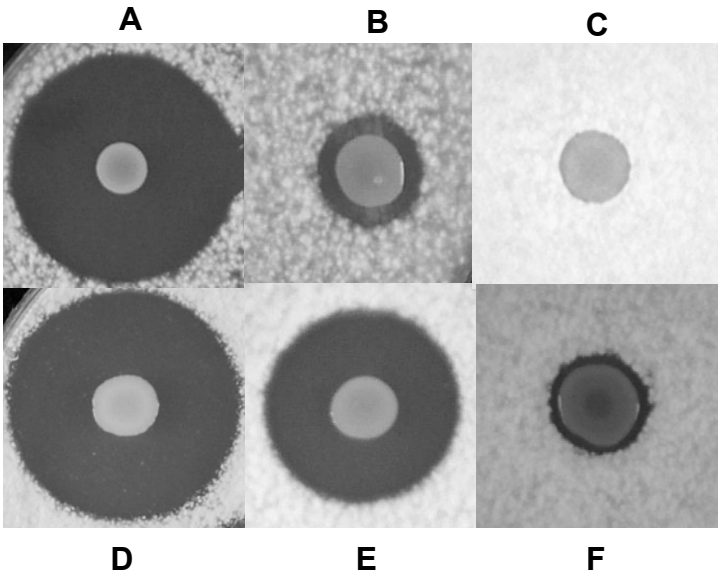


FIG. 4.4. Restoration of syringomycin production of B301DSL1 (a *syrF* mutant) and B301DNW301 (a *salA* deletion mutant) by overexpressions of the *syrF* and *salA* genes in pMEKm12. The strains were cultured 4 days on PDA agar medium supplemented with 50 μg/ml of kanamycin. The inoculated plates were oversprayed with *Geotrichum candidum*, and then further incubated overnight. To overexpress *syrF* and *salA*, plasmids pSL82 (MBP-SyrF) and pSL83 (MBP-SalA) were used, respectively. A, B301D; B, B301DSL1 (pMEKm12); C, B301DNW301 (pMEKm12); D, B301D (pMEKm12); E, B301DSL1 (pSL82); F, B301DNW301 (pSL83).

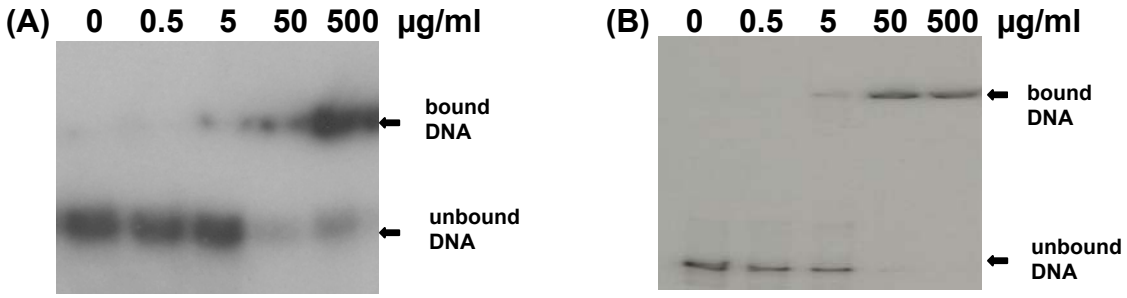


FIG. 4.5. Gel electrophoretic mobility shift analysis of the binding of SalA and SyrF with the regulatory regions of various genes. The regulatory regions of target genes were amplified by PCR and end-labeled with gamma-^{32}P ATP. The end-labeled probes were incubated with the purified MBP-SalA and MBP-SyrF proteins at the indicated concentrations, and subjected to nondenaturing polyacrylamide gel electrophoresis. (A) The 324-bp fragment (5nm) containing the potential promoter region of *syrF* was incubated without or with increasing concentrations of MBP-SalA. (B) The 262-bp fragment (5 nm) containing the promoter regions of *syrB1* and *syrP* was incubated without or with increasing concentrations of MBP-SyrF.

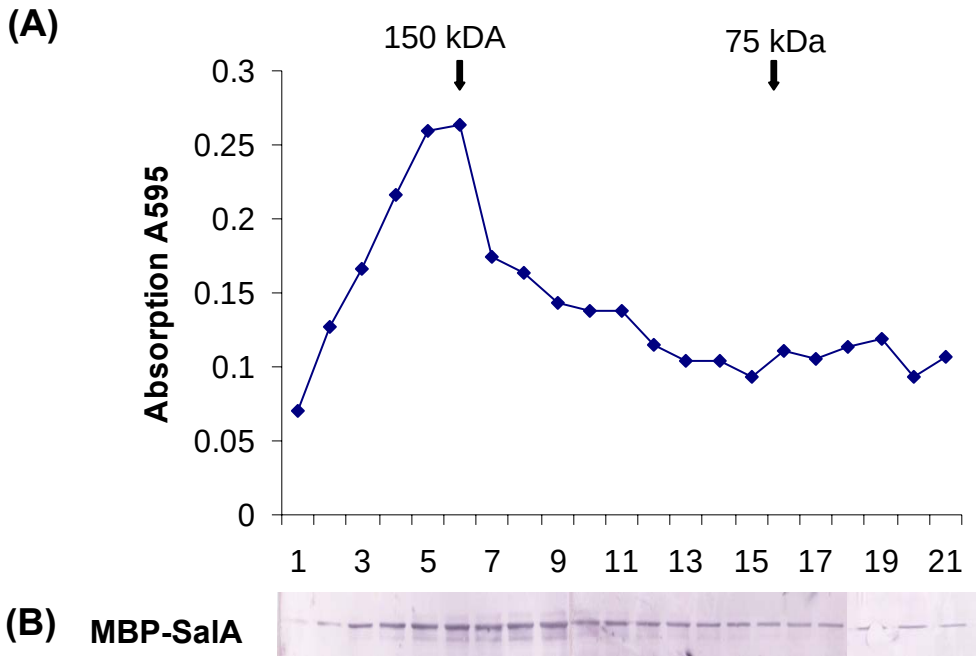


FIG. 4.6. Gel filtration of MBP-SalA protein. Purified MBP-SalA with maltose affinity chromatography were separated by gel filtration on a Sephacryl S-200 Column and analyzed by SDS-PAGE and Western blot using polyclonal antisera with antibodies for MBP. (A) Content of MBP-SalA was estimated by absorption at 595 nm using the Bradford assay. (B) The fractions obtained from gel filtration were analyzed with western blotting.

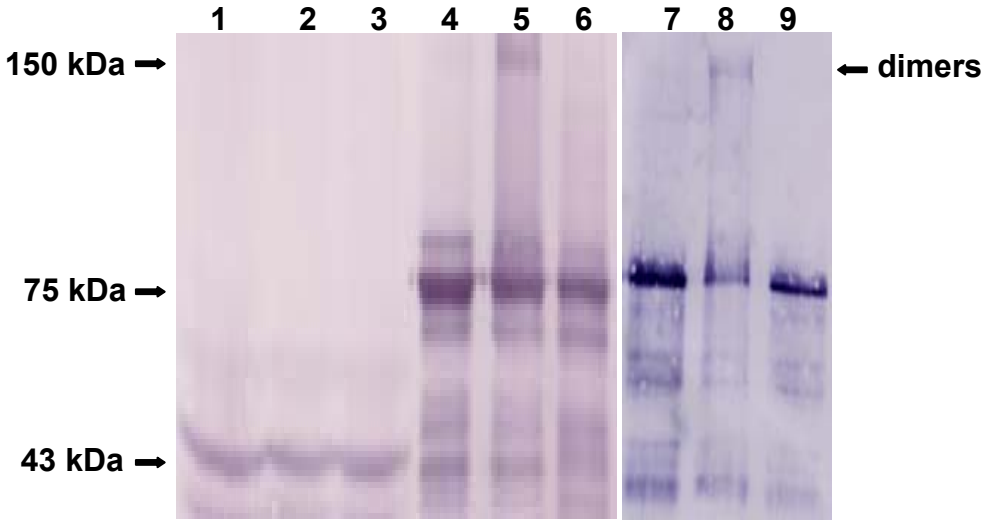


FIG. 4.7**.** In vitro cross-linking of MBP, MBP-SalA and MBP-SyrF proteins. Lane 1, MBP without cross-linking; lane 2, MBP with cross-linking; lane 3, MBP treated at 95°C for 20 min after cross-linking; lane 4, MBP-SalA without cross-linking; lane 5, MBP-SalA with cross-linking; lane 6, MBP-SalA treated at 95°C for 20 min after cross-linking; lane 7, MBP-SyrF without cross-linking; lane 8, MBP-SyrF with cross-linking; lane 9, MBP-SyrF treated at 95°C for 20 min after cross-linking. The resulting proteins were resolved on 10% SDS-PAGE, transferred to Hybond-P PVDF membrane, and immuno-blotted using polyclonal antibody to MBP, and MBP-SyrF, respectively.

and 75%, respectively (Fig. 4.4).

Interactions of the SalA and SyrF proteins with the *syr-syp* promoter regions

Once it was known that MBP fusions do not affect the function of SalA and SyrF, the purified MBP-SalA and MBP-SyrF fusion proteins were used to study the interactions between the SalA or SyrF proteins and the *syr-syp* promoter regions. Purified MBP-SyrF caused a single band shift when a 262-bp DNA fragment containing the putative promoter regions of *syrB1* and *syrP* (Fig. 4.1) was incubated in the presence of increasing concentrations of MBP-SyrF (Fig. 4.5). The retarded band was lost in competition assays in which 500-fold more unlabeled probe was used (data not shown). Purified MBP-SalA did not cause a band shift of the same 262-bp DNA fragment (data not shown), but it caused retardation of a 324-bp DNA fragment containing the intergenic region of *syrE* and *syrF* (Fig. 4.5).

Both SalA and SyrF form homodimers in vitro

Western blot analysis of the fractions obtained through maltose affinity chromatography revealed that one MBP-SalA-containing peak corresponded to a molecular mass of about 150 kDa (Fig. 4.6). This peak contained the majority of the MBP-SalA protein in a dimerized state. Cross-linking assays with purified MBP-SalA indicated that MBP-SalA forms a dimer, which migrated as an approximately 150-kDa fragment and was distinguishable from the 75-kDa fragment of the MBP-SalA monomer (Fig. 4.7). No dimers were observed when only MBP were subjected to cross-linking assays (Fig. 4.7). Similarly, dimers about 150 kDa was observed for MBP-SyrF after cross-linking. The disappearance of the dimers upon boiling (Fig. 4.7, lane 6 and 9)

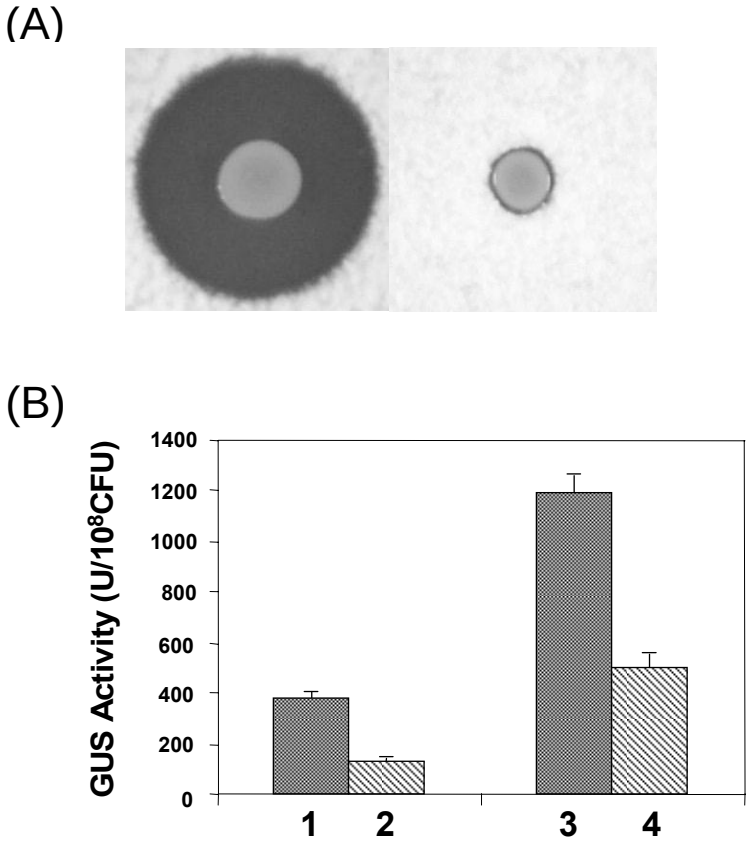


FIG. 4.8. Effect of overexpression of N-terminal region (NTR) of SalA on syringomycin production by B301D and expression of the *syrB1*::*uidA* and *sypA*::*uidA* reporters. (A) The strains were cultured on PDA media supplemented with 50 µg/ml of kanamycin for 4 days. The inoculated plates were oversprayed with *Geotrichum candidum*, and then further incubated overnight. Left: B301D (pUCP26); right: B301D (SalANTR-pUCP26). (B) Strains were incubated for 72 h at 25°C in potato-dextrose broth medium and tested for β-Glucuronidase activity (GUS): 1, B301DSL8 (pUCP26); 2, B301DSL8 (SalANTR-pUCP26); 3, B301DSL29 (pUCP26); 4, B301DSL29 (SalANTR-pUCP26). Vertical bars indicate standard error of the means for triplicate cultures.

demonstrated that the formation of the dimers was indeed the result of cross-linking by 1% formaldehyde.

Overexpression of the N-terminal region of SalA

Overexpression of the N-terminal region of SalA in B301D decreased the size of the syringomycin inhibitory zones to *G. candidum* from 16 mm to 1 mm (Fig. 4.8). In contrast, the presence of the empty pUCP26 vector in B301D had no effect on syringomycin production (Fig. 4.8A). Expression of the *syrB1*::*uidA* reporter in strain B301DSL8 was reduced from 1196 U/10^8 CFU to 501 U/10^8 CFU, a 58% decrease, by overexpression of the N-terminal region of SalA (Fig. 4.8B). Similarly, expression of the *sypA*::*uidA* reporter decreased from 381 U/10^8 CFU to 131 U/10^8 CFU, a 66% reduction (Fig. 4.8B). Both toxin bioassays and GUS assays were performed in triplicate with consistent results.

DISCUSSION

Previous studies demonstrated that *syr-syp* genes are coordinately controlled by a complex regulatory cascade including GacS/GacA (75), SalA (88, 101), and SyrF (101). This study established that both SalA and SyrF function similarly as LuxR proteins in forming dimers and by interacting with the promoter region of the target genes, the *syr-syp* genes, based on the following evidence: (i) Analysis with a subgenomic 70-mer oligonucleotide microarray, along with QRT-PCR and GUS assays indicated the *syr-syp* genes responsible for biosynthesis and secretion of syringomycin and syringopeptin belong to the SyrF regulon; (ii) Gel mobility shift analysis showed that purified MBP-SyrF, but not the MBP-SalA fusion protein, bound to a 262-bp fragment

containing the *syr-syp* box; (iii) Purified MBP-SalA caused the shift in mobility of a 324-bp band containing the putative *syrF* promoter; (iv) Gel filtration analysis and cross-linking experiments revealed that both SalA and SyrF formed dimers in vitro; and (v) Syringomycin production by B301D was decreased and GUS activities of the *sypA*::*uidA* and *syrB1*::*uidA* reporters were reduced 58% and 66%, respectively, by overexpression of the N-terminal region of SalA. This study provides a valuable foundation for understanding the regulatory mechanism of a unique subfamily of LuxR proteins.

Microarray analysis is very powerful in identifying specific regulons (19). The SyrF regulatory protein is known to be required for syringomycin and syringopeptin production based on bioassays (101). Apparently, SyrF controls all the *syr-syp* genes responsible for synthesis and secretion as a transcriptional activator as demonstrated by analysis with 70-mer oligonucleotide subgenomic microarrays (Table 4.3). This is consistent with the fact that most LuxR-type regulators act as transcription activators (53). All the *syr-syp* genes involved in synthesis and secretion of syringomycin and syringopeptin are clustered together and belong to the SyrF and SalA regulons (Fig. 4.2). Cluster analysis has been widely used to identify co-regulated genes and to define regulons (174). Apparently, *salA*, *syrF* and *syrG* do not belong to the SyrF regulon because mutation of *syrF* has no effect on GUS activities of the *salA*::*uidA*, *syrF*::*uidA* and *syrG*::*uidA* reporters (101). As reported previously (101), *salA* positively controls *syrF*. Cluster analysis (Fig. 4.2) and GUS assays indicate that the difference between the SalA and SyrF regulons is that SalA controls the *syrF* gene and the SyrF regulon. SalA controls the *syr-syp* genes through SyrF because gel shift analysis demonstrates that

SalA binds a 324-bp DNA fragment containing the putative *syrF* promoter region but not a 262-bp DNA fragment containing the potential promoter regions of *syrB1* and *syrP* (Wang and Gross unpublished data).

SalA and SyrF resemble other LuxR proteins in regulation by binding to the promoter region of target genes (53, 101). Purified MBP-SyrF binds to a 262-bp fragment containing a 20-bp sequence with dyad symmetry (TGTCccgN4cggGACA, termed the *syr-syp* box) overlapping with the -35 region of *syrB1* (Fig. 4.5) (Wang and Gross unpublished data). The *syr-syp* box is required for expression of the *syrB1*::*uidA* fusion and is identified in the promoter regions of the *syr-syp* genes/operons responsible for biosynthesis and secretion of syringomycin and syringopeptin (Wang and Gross unpublished). It is possible that SyrF binds to the *syr-syp* box even though no direct evidence of binding of the inverted repeats is available. This is true for LuxR since LuxR binds to the *lux* box, which is 20-bp in length with a dyad symmetry centered at the -42.5 position relative to the transcriptional start site of *luxI* (43). In addition, the HTH domains of SyrF and LuxR share significant homology with the 2.4 region of σ^{70} subunit of RNA polymerase, which interacts with the -35 region (101, 122). MBP-SalA binds to a 324-bp fragment containing the potential *syrF* promoter region (Fig. 4.5). Unlike LuxR, the binding of purified MBP-SalA and MBP-SyrF with a target DNA sequence does not require the binding of an autoinducer. Most LuxR autoinducer-binding proteins are involved in quorum sensing to respond to cell population density by binding to acyl-homoserine lactone signal molecules and control the regulation of gene expression in Gram-negative bacteria (54). Purified LuxR binds specifically to DNA

containing a *lux* box in the presence of N-(3-oxohexanoyl) homoserine lactone (3OC6-HSL) (162). Binding of 3-oxo-octanolyl-HSL (3OC8-HSL) is required for the interaction of TraR and the *tra* box, an 18-bp palindromic element, in *Agrobacterium tumefaciens* (182). In vitro expression of TraR without autoinducer does not bind to the *tra* box (130). The fact that autoinducer is not required for the binding of SalA and SyrF to the target DNA sequence can be explained by the fact that both SalA and SyrF do not contain acyl-HSL autoinducer-binding domain and acyl-HSL is not required for the function of both SalA and SyrF. This is consistent with the fact that mutation of *ahlI*, which is responsible for the production of acyl-HSL, did not have effect on production of syringomycin (Wang and Gross unpublished data). NarL and NarP, which are response regulators that belong to the LuxR family, interact with heptamer sequences that are often present as pairs of inverted repeats, in the promoter regions of the target genes (39, 107). Phosphorylated FixJ, a response regulator, binds to target DNA with high affinity (38). It remains unknown about the phosphorylation status of functional SalA and SyrF proteins and whether they are activated by phosphorylation as described for the response regulator NarL (179) or by some unknown signal molecule.

Dimerization is critical for transcriptional factors such as TraR (104) and NarL (107) to bind to promoter regions of target genes. Both MBP-SalA and MBP-SyrF resemble LuxR proteins in dimerization. Both SalA and SyrF form dimers in vitro as evidenced by gel filtration and cross-linking analyses (Fig. 4.6). Syringomycin production of B301D and expression of the *syrB1*::*uidA* and *sypA*::*uidA* reporters were decreased by overexpression of the N-terminal region of SalA (Fig. 4.8). This result can

be explained by the fact that the N-terminal region of SalA is responsible for dimerization. Consequently, overexpression of the N-terminal region results in the formation of a nonfunctional heterodimer that cannot bind to the promoter region. Overexpression of the N-terminal domain of LuxR interferes with the luminescence (29). Residues between 116 and 161 in the N-terminal domain are critical for oligomerization of LuxR (29). In addition, the N-terminal domain of TraR (residues 119-156) is required for dimerization, which is requisite for binding of TraR to the *tra* box (104). TrlR, a truncated TraR homolog lacking the C-terminal HTH DNA-binding domain, inhibits the function of TraR by forming an inactive heterodimer (181). The dimerization of SyrF, LuxR (29), and TraR (130) is consistent with the existence inverted repeat sequences in the promoter regions of *syr-syp* genes (Wang and Gross unpublished data), *luxI* (43), and *tra* operon (182). It is postulated that the dimer binds to a particular DNA sequence near target promoters to activate transcription (54). Unlike LuxR and TraR, purified CarR in *Erwinia carotovora* forms a dimer in cells grown without the acyl-HSL (168). The acyl-HSL determines the specific binding of CarR to the promoter region of the target genes (168). Response regulators, such as NarL and FixJ, form dimers in the presence of phosphorylation signals, but not in the presence of an autoinducer (38, 107). These data clearly indicate that SalA and SyrF function similarly as LuxR proteins even though they are not activated by autoinducers and their phosphorylation status remains unknown (101).

Plasmid pMEKm12 is an appropriate shuttle vector to overexpress proteins in both *E. coli* and *P. syringae* (101). Plasmid pMEKm12 contains the *malE* gene, which

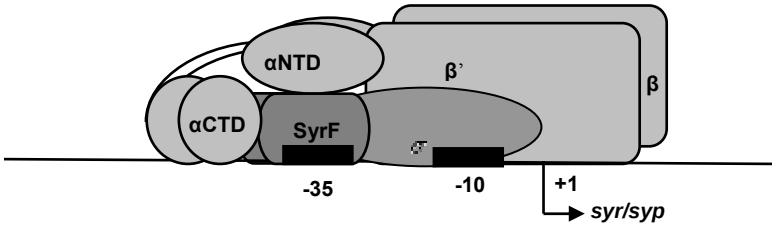


FIG. 4.9. A model for SyrF-dependent transcription of the *syr-syp* genes. The SyrF protein binds to the *syr-syp* box, which overlaps with the –35 promoter region of *syr-syp* genes, acting as a Class II activator. SyrF contacts both the sigma subunit of an unknown member of the sigma 70 family and the α-subunit carboxy-terminal domain (αCTD) of RNA polymerase to recruit RNAP to the *syr-syp* promoter regions and directs transcription of both syringomycin and syringopeptin synthetase and secretion genes by binding to the –10/–35 promoter regions.

encodes a maltose-binding protein (MBP). The MBP is fused to the overexpressed protein and increases stability of the overexpressed proteins. This character is critical for the expression of bacterial regulatory proteins since many bacterial proteins are unstable to cytoplasmic proteases (182). The pMEKm12 was successfully used to overexpress the syringomycin synthetase SyrB1 and expression of the MBP-SyrB1 protein restored syringomycin production of a *syrB1* mutant BR132A1 (101). Similarly, the overexpressed MBP-SalA and MBP-SyrF are functional since they complemented the *salA* mutant B301DSL7 and the *syrF* mutant B301DSL1, respectively, to produce syringomycin (Fig. 4.4). Therefore, the MBP fusion protein does not abolish the function of the fusion proteins MBP-SalA and MBP-SyrF. It is expected that overproduced SalA and SyrF in *E. coli* will be functional biologically. Several regulatory proteins such as LuxR were successfully expressed in *E. coli* without interfering with their function (45).

In conclusion, both SalA and SyrF are similar to LuxR proteins in dimerization and by interaction with promoter regions of target genes. SalA regulates the *syr-syp* genes by forming a dimer and interacting with the *syrF* promoter. The N-terminal region of SalA is responsible for dimerization. Subsequently, as proposed in the model shown in Fig. 4.9, SyrF forms a dimer and activates the *syr-syp* genes responsible for synthesis and secretion by binding to the promoter regions. SyrF interacts with sigma 70 factor and αCTD of RNA polymerase to recruit RNAP to the *syr-syp* promoters as LuxR (101). LuxR contacts both the sigma 70 factor and αCTD of RNA polymerase to recruit RNAP to the *luxI* promoter (155). This is consistent with the fact that sigma 70

dependent promoters were identified for the *syr-syp* genes. In addition, the second primary sigma factor sigma 38 that binds to similar promote sequence as sigma 70 is not involved in syringomycin and syringopeptin production (Wang et al. unpublished data). Thus, results of this study provide evidence that SyrF is the key transcriptional factor in activation of the *syr-syp* genes for *P. syringae* pv. *syringae* to adapt to the rapidly changing environment. This study is the first report to delimit the regulatory mechanism of a unique LuxR subfamily of proteins.

CHAPTER V

CONCLUSIONS

The regulation of syringomycin and syringopeptin production by *P. syringae* pv. *syringae* is complex. It has been known that plant signal molecules induce syringomycin production (115, 131). Expression of *syrB1*, a syringomycin synthesis gene, is specifically induced by phenolic glucosides and enhanced by sugars such as D-fructose and sucrose (115). Syringomycin production is also controlled by GacS/A, SalA, and SyrF (88, 101). However, despite of these progresses made in understanding the regulation of syringomycin production, the regulation of syringopeptin production remains largely unknown. Hence the main focus of my study was to understand whether plant signal molecules induce syringopeptin production; how plant signals induce the *syr-syp* genes; to characterize the common features of the *syr-syp* gene promoters; to determine the function of SalA and SyrF in syringomycin and syringopeptin production.

Plant signal molecules induce syringopeptin production of different strains of *P. syringae* pv. *syringae* as evidenced by bioassay with *Bacillus megaterium* Km as indicator. The induction of syringopeptin production resembles that of syringomycin production. Phenolic glucosides such as arbutin and salicin activate transcriptional expression of the *sypA*::*uidA* reporter based on GUS assays. Plant signals induce the coordinated expression of the *syr-syp* genes involved in synthesis, secretion, and regulation of syringomycin and syringopeptin production as demonstrated by analysis of 70-mer subgenomic microarrays. Microarray analysis clearly demonstrates that the

bacterium coordinate expression of the *syr-syp* genes and representative genes associated with the Type III secretion system in response to plant signal molecules (Fig. 2.5).

The induction of plant signals on the *syr-syp* genes is mediated by the regulatory cascade including GacS/GacA, SalA and SyrF. The *syr-syp* genes induced by arbutin and D-fructose are clustered together and belong to the SalA and SyrF regulons as defined by cluster analysis, GUS assays and bioassays. The *salA* and *syrF* mutants failed to produce syringomycin and syringopeptin as shown by bioassay on SRM and SRM_{AF} media. As demonstrated with QRT-PCR, plant signal molecules do not activate the expression of the *syr-syp* genes in a *gacS* mutant, while the induction of the *hrp* genes is not affected by mutation of *gacS*. This indicated that the plant signal induction of syringomycin and syringopeptin in B301D is via the GacS pathway, while the induction of the *hrp* genes uses a different pathway.

The *syr-syp* genes share high similarity in their promoters. The *syr-syp* genomic island is divided into five operons and seven individual genes. Consequently, the *syr-syp* genomic island contains at least 12 promoters. The promoters of operons III, IV and *salA* gene have been experimentally defined based on primer extension. The -10/-35 region of operon IV (*syrB1-syrB2-syrC*) was determined by deletion and site-directed mutagenesis analysis. The promoters of the *syr-syp* genes/operons share high similarity with sigma 70 factor dependent promoters. What's more, the *syr-syp* box (TGTCccgN4cggGACA), a 20-bp sequence with imperfect dyad symmetry has been identified as required for expression of the *syrB1*::*uidA* reporter. Similar sequences were observed for the *syr-syp* genes/operons responsible for synthesis and secretion of

syringomycin and syringopeptin. The high similarity in the promoters of the *syr-syp* genes/operons attribute to the co-regulation of syringomycin and syringopeptin.

The regulatory effect of *salA* is through *syrF*. SalA positively controls the expression of *syrF* (Lu et al. 2002). There is no production of syringomycin and syringopeptin by a *syrF* mutant. Mutation of *syrF* causes coordinated decrease in expression of the *syr-syp* genes responsible for synthesis and secretion as defined by microarray analysis. The difference between SalA and SyrF regulons is that SalA regulons controls *syrF* and the SyrF regulon.

Both SalA and SyrF resemble LuxR proteins in dimerization and interaction with the promoter regions of the target genes. SalA regulates the *syr-syp* genes by forming dimer and interacting with the *syrF* promoter. The N-terminal region of SalA is responsible for dimerization. As proposed in the model shown in Fig. 4.9, SyrF forms dimer and activates the *syr-syp* genes responsible for synthesis and secretion by binding to the promoter regions and interacts with sigma 70 factor and αCTD of RNA polymerase to recruit RNAP to the *syr-syp* promoters.

This work improved our understanding of regulation of syringomycin and syringopeptin production by *P. syringae* pv. *syringae*. Some of the highlights of this work have been identification of members of the stimulon of plant signal molecules and the SyrF regulon, identification of the -10/-35 of *syrB1*, identification of the *syr-syp* box, characterization of the dimerization status of SalA and SyrF and their interaction with the promoter region of the target genes, the *syr-syp* genes. This work help us in the understanding the regulatory network that controls phytotoxin production in

microorganisms, which is poorly understood, and provides valuable foundation for understanding a unique LuxR subfamily regulatory proteins. Future work will determine the mechanism of GacA controlling the *salA* gene, the interaction of SalA and SyrF with the promoter regions using purified native proteins in full length expressed in B301D, the critical domains of SalA and SyrF, and the interaction of SalA and SyrF with the RNA polymerase.

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